A TRANSLATION STRATEGY AND GENE MAP FOR

PEA SEEDBORNE MOSAIC VIRUS

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"When you have eliminated
the impossible, whatever
remains, however
improbable, must be
the truth."

Sir Arthur Conan Doyle
The Sign of Four

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ABBREVIATIONS

aa	amino acid
AI	amorphous inclusion
AlMV	alfalfa mosaic virus
APS	ammonium persulphate
ATP	adenosine triphosphate
BMV	brome mosaic virus
bp	base pairs
BPB	bromophenol blue
BSA	bovine serum albumin
BYMV	bean yellow mosaic virus
CaMV	cauliflower mosaic virus
CPMV	cow pea mosaic virus
CCMV	cowpea chlorotic mottle virus
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal alkaline phosphatase
CI	cytoplasmic inclusion
CMV	cucumber mosaic virus
CPMV	cow pea mosaic virus
CP	coat/capsid protein
dd/dATP	dideoxy/deoxyadenosine triphosphate
dd/dCTP	dideoxy/deoxycytidine triphosphate
dd/dGTP	dideoxy/deoxyguanosine triphosphate
dd/dTTP	dideoxy/deoxythymidine triphosphate
DEPC	diethylpyrocarbonate
DIECA	diethyldithiocarbamic acid
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTE	dithioerythritol
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbant assay
HC	helper component
IPTG	isopropylthiogalactoside
kDa	kilodaltons

kb	kilobases
MDL	messenger dependent lysate
M-MLV	murine moloney leukemia virus
NI	nuclear inclusion
PEG	polyethylene glycol
PeMV	pepper mottle virus
poly (A)	polyadenylate
PPV	plum pox virus
PRV	papaya ringspot virus
PTA	phosphotungstic acid
PVY	potato virus Y
RNA	ribonucleic acid
SCMV	sugarcane mosaic virus
SDS	sodium dodecyl sulphate
SMV	soybean mosaic virus
TCA	trichloroacetic acid
TEM	transmission electron microscopy
Temed	N,N,N',N'-tetramethylethylenediamine
TEV	tobacco etch virus
TMV	tobacco mosaic virus
TRV	tobacco rattle virus
TuMV	turnip mosaic virus
TVMV	tobacco vein mottling virus
VPg	viral genome linked protein
Xgal	5-bromo-4-chloro-3-indoyl-b-D-galactoside

ABSTRACT

A method was developed to purify pea seedborne mosaic virus (PSbMV) and its genomic RNA from peas. The RNA of PSbMV was found to be polyadenylated and approximately 10,000 nucleotides long. A cDNA library of 360 clones was made. Of these, 80 clones were screened to determine the size of the inserts. One or more *Rsa*I fragments were sequenced from each of three clones, pPSb54, pPSb66 and pPSb401. A fourth clone, pPSb70, was used as a probe in Northern blots.

The cylindrical inclusions of PSbMV were found to have several different morphologies. Pinwheels, scrolls and short laminate aggregates were all observed to be induced by PSbMV. The presence of all three types of inclusions places PSbMV in subdivision-IV according to the classification of Edwardson *et al.* (1984).

The molecular weights of 3 virus-encoded proteins were established by Western blots. The molecular weights are: coat protein 33 kDa, nuclear inclusion b protein 48 kDa, and the cylindrical inclusion protein 70 kDa. The molecular weight of the NIa protein (45 kDa) was established by immunoprecipitation of *in vitro* translation products.

A gene map for PSbMV was proposed, using information from serologically cross-reacting products of the *in vitro* translations of PSbMV-RNA together with information from Western blots. The proposed map places the coat protein gene at the 3' end of the genome. Upstream of this is the putative replicase gene (NIb), then a putative protease (NIa), followed by the cylindrical inclusion gene. The gene encoding the amorphous inclusion protein is nearer the 5' end.

The translation strategy of PSbMV was determined using *in vitro* translation of PSbMV-RNA in rabbit reticulocyte lysate. The virus produces a polyprotein that is proteolytically cleaved to produce 7 or more mature products. Full length PSbMV-RNA was isolated from the polyribosomes of infected plant material.

The amount of PSbMV-RNA electroporated into the protoplasts of *Nicotiana* species was found to increase very rapidly for the first 4 hours of incubation, then the RNA level was maintained for a further 8 hours. After 12 hours incubation, the amount of RNA was observed to decrease. Western blots using antiserum to the coat protein of PSbMV indicated that PSbMV-RNA was translated in *Nicotiana tabacum* protoplasts.

CHAPTER 1

INTRODUCTION

The RNA viruses are the most ubiquitous and successful parasites known. An estimated 94% of all characterised plant viruses have RNA-based genomes. Of these, 83% are single stranded, positive sense RNA viruses (Hull and Davies 1983).

The potyvirus family is the largest known group of plant viral pathogens. The group contains over 100 different members depending upon the criterion used to define a distinct virus (Edwardson *et al.* 1984; Hollings and Brunt 1981). Potato virus Y (PVY) is the type member for the group. Members of the potyvirus family have a single-stranded, polycistronic RNA genome of positive sense (Allison *et al.* 1986; Domier *et al.* 1986). The RNA molecules are approximately 10,000 nucleotides in length (Allison *et al.* 1986; Domier *et al.* 1986) and are polyadenylated at the 3' end (Hari *et al.* 1979). The 5' ends are covalently linked to a small viral genome-linked protein (VPg) (Hari 1981; Shahabuddin *et al.* 1988). The RNA of several members, including wheat streak mosaic virus (Brakke and van Pelt 1970), has been shown to be infectious without the coat protein. The genomes of tobacco etch virus (TEV) and tobacco vein mottling virus (TVMV) are predicted to code for 8 mature peptides (Dougherty and Carrington 1988). One of the mature peptides is the coat protein which packages the RNA into long flexuous rods 680-900 nm in length and 11-15 nm wide (Hollings and Brunt 1981). The RNA comprises approximately 5% of the particle (Matthews 1982).

All members of the potyvirus group encode a protein that aggregates in the cytoplasm of infected cells to form pinwheel or scroll-shaped inclusion bodies (Christie and Edwardson 1977; Edwardson 1974). The cytoplasmic "cylindrical inclusions", as they are known, are a diagnostic feature of potyvirus infections.

Recent comparisons between homologous non-structural proteins of viruses from different families indicate that potyviruses have a counterpart with an animal virus family, the picornaviruses and also with the plant virus families como- and caulimo- viruses (Domier *et al.* 1987). These families share many common features which are discussed in Chapter 5.

Potyviruses are usually transmitted by aphids in a nonpersistent, noncirculative manner (Govier *et al.* 1977; Govier and Kassanis 1974). They may also be transmitted by seed [as in the case of pea seedborne mosaic virus (PSbMV)], by mites (Hollings and Brunt 1981), or by Mechanical inoculation.

PSbMV is a member of the potyvirus family. The virus was first discovered in 1966 in Czechoslovakia, where it was known as pea leaf rolling virus (Musil 1966, cited in Khetarpal and Maury 1987). Since then, the virus has had a number of different names; for example, pea frizzle top virus (Hampton 1969), pea leaf roll mosaic virus (Bos 1970), and "a seedborne virus of pea"

(Mink *et al.* 1969). The name 'pea seedborne mosaic virus' was proposed by Mink *et al.* (1974) to include all the above viruses, which are principally seed-transmitted.

PSbMV is a pathogen of considerable importance. The virus can infect 47 species in 12 dicotyledonous families (Aapola *et al.* 1974). The main hosts, however, are cultivars of *Pisum*. *Pisum sativum* L. (peas) are grown all over the world and rank next to cereals and starch crops in importance as a source of food. Yield reductions of 28% have been reported in peas infected with PSbMV (Maury and Bossennec, unpubl. in Khetarpal and Maury 1987). One of the reasons PSbMV is a problem is that it is transmitted through seed. Commercial seed lots of peas have been reported to contain 90% infected seed in the USA (Mink *et al.* 1969; Knesek and Mink 1970). Transmission of the virus through seed, coupled with the ready exchange of seed between countries and the lack of a reliable test for the detection of PSbMV, has meant that infected seed has been disseminated all over the world.

Much of the information that is available on PSbMV is of a general biological nature. Knesek *et al.* (1974) produced much of the early molecular information. Their report established; (1) a sedimentation coefficient of 148 S for the virus; (2) the particles are composed of 5.3% RNA; (3) the base ratio is adenine 44.0%, guanine 22.8%, cytidylic acid 17.6%, and uridylic acid 15.6%; (4) the coat protein contains aspartic acid and glutamic acid in the greatest amounts and (5) the modal length of the particle is 770 nm.

My study expands our knowledge of PSbMV at a molecular level.The thesis is presented as a series of chapters, each with its own introduction, methods, results, and discussion sections. The chapters are not independent and each relies on information and deductions from other chapters. In Chapter 2, the purification of PSbMV and the isolation of RNA is discussed. The constructing of a cDNA library and sequencing of fragments of the library are discussed.

The types and morphologies of the various inclusion bodies induced by PSbMV in infected cells are investigated in Chapter 3. The potyvirus family is such a large group that further subdivision is desirable. The place of PSbMV in one of the subdivisions suggested by Edwardson (1974) is discussed.

The technique of Western blots is used in Chapter 4 to establish the molecular weights of some of the virus-encoded proteins. Only the molecular weight of the coat protein of PSbMV has been previously established (Huttinga 1975).

Chapter 5 discusses the products of the *in vitro* translation of PSbMV-RNA in messenger-dependent rabbit reticulocyte lysate. The products of the translations were immunoprecipitated with antisera to several virus-encoded proteins. The information collected from a number of different sources was compiled to propose a gene map for PSbMV and is used to discuss a translation strategy for this virus.

In the final experimental chapter (Chapter 6), the technique of electroporation is used to introduce both viral RNA and whole virus into protoplasts of *Nicotiana* species.

The final chapter (Chapter 7) summarises the molecular information gained for PSbMV in this study. It also suggests some of the possible directions that further investigations, both of PSbMV and the potyvirus family as a whole, may take.

CHAPTER 2

GENERAL METHODS, VIRUS PURIFICATION, RNA ISOLATION, PRODUCTION OF CDNA CLONES AND SEQUENCING

2.1 INTRODUCTION

Potyviruses have been notoriously difficult to purify, presenting researchers with a variety of problems. One of the more serious obstacles encountered is aggregation of the virus particles during purification (de Bokx and Huttinga 1981). This leads to high losses during low speed centrifugation, which presents a problem for a virus already reputed to be in low concentrations in infected plant material (Stevenson and Hagedorn 1973). Stevenson and Hagedorn also reported the virus particles adhered to plant tissue, causing further losses during low speed centrifugation. Inouye (1967) observed that PSbMV is sensitive to a variety of solvents used to clarify the initial extract. All these problems had to be overcome to purify PSbMV successfully.

The greatest problem encountered in isolating full length potyviral RNA, additional to the ever present problem of RNase, is shearing of the RNA, probably when it is still encapsidated. The 10 kb, single-stranded molecule is susceptible to breakage (Dougherty 1983). Also it appears that there is a "critical mass" factor involved; unless RNA isolation begins with more than 5 mg of pure virus, no RNA is isolated (R.Forster pers. comm.) probably due to losses occurring during the various purification steps. Obtaining a large enough quantity of virus is a problem when the low concentration of virus in host tissue and low yields from purifications are considered. Full length RNA is necessary to program the *in vitro* translations which are an essential part of this study.

The first use of the enzyme reverse transcriptase to produce cDNA from an RNA template for molecular cloning was achieved by Rougeon and Mach (1976). The production of cDNA from plant viral RNA followed and demonstrated the wide variety of applications this technique made available, some of which are listed below. Allison *et al.* (1986) produced the first full length nucleotide sequence of a potyvirus, TEV. This was soon followed by the sequence of TVMV (Domier *et al.* 1986). Both studies used cDNA as an intermediate step to sequencing. Dougherty et al. (1985) used cDNA clones to PeMV in gene mapping experiments using hybrid-arrest translations. Abu-Samah and Randles (1981) made cDNA probes to BYMV to investigate the relationships between 3 isolates of the virus. In my study, a cDNA probe to the coat protein region of PSbMV was used to probe Northern blots (see Chapter 5). Another important application of virus directed cDNA is "genetically engineered cross-protection" (Powell Abel *et al.* 1986).

The common method for cDNA production (Okayama and Berg 1982) was modified by Gubler and Hoffman (1983) to combine oligo-(dT) primed first-strand synthesis with RNase H and DNA polymerase I for the production of the second strand. D'Alessio *et al.* (1987) adapted the method to use only a single tube.

A pUC series plasmid (pUC19) developed by Yanish-Perron *et al.* (1985) was used in this study. The plasmids have the advantages of high copy number, small size and the ability to integrate up to 20 kb of foreign DNA into a genetically engineered polylinker in the β-galactosidase gene. The artifical chromogenic substrate 5 bromo-4-chloro-3-indole-β-D-galactopyranoside (Xgal) produces a marked colour difference between *E.coli* transformed by plasmids containing inserts (white colonies) and those without inserts (blue colonies). Alternative systems use bacteriophages, usually series lambda, cosmids or other plasmids.

Sequence data from potyviruses have been used in a number of ways. For example, Shukla and Ward (1988) have attempted to address the problem of classifying potyviruses by comparing the protein sequence at the amino termini of potyvirus coat proteins. Nucleotide sequence data provide information on other questions; for example, a tentative function has been assigned to the product of the NIb gene in potyviruses based on sequence similarities to a gene found in picornaviruses (Domier *et al.* 1987). The product of the picornavirus gene is known to function as an RNA-dependent RNA-polymerase. On the other hand, analysis of nucleotide sequence data has given rise to confusion in some instances. For example, on the basis of sequence data Dougherty *et al.* (1985) proposed that the coat protein of PeMV was encoded by a sgRNA. This has since been proven to be incorrect (Dougherty and Carrington 1988). These and other answers have become possible only with the development of cDNA cloning and subsequent sequencing.

In this chapter, the methods for the purification of PSbMV and the isolation of its RNA are discussed. The production of a cDNA library and the analysis of some fragments of the clones produced are also discussed. The use of oligo-(dT) as a primer for first strand synthesis in a group of viruses known to be adenine-rich (Hill and Benner 1976) is investigated. The use of a cloning method where linkers are not used is also considered. Here I assume the cDNA produced using DNA polymerase I in the second strand reaction has a predominance of blunt ends. This reasoning is supported by the use of this enzyme to "end fill" DNA (Maniatis *et al.* 1982). The cDNA can then be ligated into blunt ended vectors without the use of linkers. This chapter also deals with the suitability of these clones for use as probes for sgRNA in Northern blotting experiments (see Chapter 5) and for the production of a clone suitable for detecting PSbMV infections using hybridisation techniques (see Chapter 6) (Hull and Al-Hakim 1988; Maule *et al.* 1983). This would be highly desirable, as the ELISA test, which is currently the most widely used diagonostic test for PSbMV, can produce unreliable results in the detection of PSbMV infections (A. Russell pers. comm.). Sequencing fragments of selected clones was undertaken

primarily to gain experience with the technique. A minor objective was to establish the position of these clones on the potyvirus genome by comparison with the sequences of TEV and TVMV (Allison *et al.* 1986; Domier *et al.* 1986).

2.2 MATERIALS AND METHODS

2.2.1 GENERAL METHODS

Ethanol precipitation (Maniatis *et al.* 1982)

Nucleic acids were precipitated from a variety of different solutions by the addition of 0.1 volumes of 3 M sodium acetate, pH 5.0, and two volumes of ice-cold 100% ethanol. These were placed at either -80°C for 10 minutes or -20°C from two hours to overnight. The pellet was collected by a 15 minute spin at 13,000 g in a microfuge. Normally the pellet was briefly dried *in vacuo*.

For quantitative recovery of less than microgram quantities, DNA was precipitated by adding 0.5 volumes of 7.5 M NH_4OAc and 2.5 volumes of 100% ethanol (Crouse and Amorese 1987). The precipitate was allowed to form overnight at 4°C and collected by centrifugation at 13,000 g in a microfuge for 30 minutes. The pellet was dried as before.

Trichloroacetic Acid Precipitation (Maniatis *et al.* 1982)

300 μl of cold 10% trichloroacetic acid (TCA) containing 10 mM sodium pyrophosphate was added to the sample (for sample volumes see section 2.2.5). This was chilled on ice for 15 minutes then washed through a Whatman GF/C glass fibre disc with 6 washes of cold 5% TCA containing 10 mM sodium pyrophosphate. The disc was dried at 80°C and added to a scintillation vial containing 5 mls of scintillation cocktail (0.1 g (1,4-bis[2-(5-Phenyloxa 2 olyl)] benzene)(POPOP), 5 g 2-5-diphenyloxazole (PPO) in 1 litre of toluene). The sample was counted in a liquid scintillation counter.

Phenol Chloroform Extraction (Maniatis *et al.* 1982)

An equal volume of phenol saturated with 0.1 M Tris-HCl, pH 7.6, was added to the sample. The mixture was vortexed and spun at 8,000 g in a microfuge for two minutes to separate the phases. The upper aqueous phase was collected and the organic phase back-extracted by the addition of an equal volume of 10 mM Tris-HCl pH 7.5, vortexed and spun as before. Again the aqueous phase was collected and pooled with the first aqueous phase. An equal volume of chloroform:isoamyl alcohol (24:1) v/v was added, followed by vortexing, and spun as before. Nucleic acids were collected by ethanol precipitation.

Agarose Gel Electrophoresis

Routine agarose gel electrophoresis was carried out in gels containing 1% (w/v) agarose dissolved in 1x TBE (0.089 M Tris Base, 0.089 M boric acid and 0.008 M EDTA). The samples were mixed with 5 x loading buffer (50% glycerol, 50% 1x TBE and 0.25% Bromophenol blue). The electrophoresis running buffer consisted of 1x TBE, and the gels were run at 15V/cm.

Preparation of Electron Microscope Grids

Plant material (0.1 g) was homogenised in 800 μl phosphate buffer (4 mM KH_2PO_4, 16 mM Na_2HPO_4 and 155 mM NaCl) pH 7.0. The sample was centrifuged at 5,000 g in a bench top microfuge for 5 minutes. Copper grids coated with formvar were dipped into a drop of the supernatant. The grids were drained and then dipped into phosphotungstic acid, pH 7.0. The grids were air dried and examined with a transmission electron microscope.

Sterilisation Procedure

All glassware and plasticware required to be sterile was autoclaved at 121°C and 15 p.s.i. for 20 minutes and then oven baked at 80°C for a minimum of 3 hours. Liquids required to be sterile were autoclaved as above, but oven treatment was eliminated. Glassware and plasticware required to be RNase free were treated by soaking overnight in 0.1% diethylpyrocarbonate (DEPC). Traces of DEPC were removed by either autoclaving the drained items followed by oven baking for a minimum of 3 hours, or rinsing the items 10 times with autoclaved DEPC treated dH_2O. In all experiments involving RNA, disposable gloves were worn.

2.2.2 Propagation and Infection of *Pisum sativum*.

Infected *Pisum sativum* (pea) plants were supplied by the Crop Research Division of the Department of Scientific and Industrial Research (DSIR), Lincoln. The plants were inoculated mechanically three times by inoculation using 400 mesh carborundum powder at intervals of approximately 3 days. These times corresponded to the 2,4,and 6 leaf stages of the pea plants. The plants were grown for 2-5 weeks, after which the shoots were harvested.

2.2.3 Virus Purification

Several different virus purification procedures were tried. The method of Hamilton and Nicols (1978) uses a high speed centrifugation, followed by a spin through a sucrose cushion to purify the virus. The method of Knesek *et al.* (1974), which uses a sucrose gradient containing polyethylene glycol (PEG) was also tried. However, modifications of the method by Reddick and Barnett (1983) were found to be the most satisfactory.

Plant material (1kg) was homogenised in a Waring blender for 2 minutes in 1.5 volumes of ice cold buffer containing 0.5 M phosphate buffer, pH 7.0, 0.14 M β mercaptoethanol, 0.17 M ascorbic acid and 0.01 M sodium DIECA. The homogenate was expressed through cheese cloth and the filtrate clarified by the addition of 0.8 volumes of chloroform. This was homogenised for a further 30 seconds, then centrifuged at 5000 g for 15 minutes. The virus was precipitated from the supernatant by the addition of 5% (w/v) PEG, MW 6000 and 0.25 M NaCl, then stirred for one hour at 4°C. The precipitated virus was collected by centrifugation at 5,000 g for 15 minutes. The resulting pellet was resuspended in 100 ml of phosphate/urea buffer containing 0.5 M phosphate, 3% urea (w/v) and 1% Igepon (v/v). The solution was stirred for two hours at 4°C, followed by centrifugation at 5,000 g for 15 minutes. The supernatant was collected and made 4% PEG/0.25 M NaCl and stirred for one hour at 4°C. The precipitated virus was collected as before. The resulting pellet was resuspended in 37.5 ml (final volume) of phosphate/urea buffer containing 7.5 g caesium sulphate (Cs_2SO_4) with the aid of a glass tissue grinder. The resuspended material was layered onto three 1.07 ml, 53% Cs_2SO_4 cushions and centrifuged at 26,000 rpm in a Beckman SW41 rotor for 16 hours at 4°C. The opalescent virus band was collected, diluted 5 times in 20 mM Tris-HCl pH 7.0, and dialysed against one litre of 20 mM Tris-HCl pH 7.0, for 4 hours with a buffer change after one hour. The dialysate was centrifuged at 8,000 g for 10 minutes and the supernatant collected. This was centrifuged at 41,000 rpm in a Beckman SW41 rotor for 100 minutes at 4°C. The pellet was recovered and resuspended in 250 μl 20 mM Tris-HCl pH 7.0.

200 pmol of virions purified as described above were resuspended in 100 μl dH_2O and sent to A. Carne, Biochemistry Department, University of Otago, for amino acid sequence analysis by automated Edman degradation.

2.2.4 RNA Isolation (Brakke and van Pelt 1970)

RNA was extracted by dissociating freshly purified virus in an equal volume of 0.2 M Tris-HCl pH 9.0, 2% sodium dodecyl sulphate (SDS), 2 mM EDTA, 100-500 μg/ml Bentonite, and 10 μg Proteinase K (predigested for 30 minutes at 37°C) per mg of virus. The sample was digested for 15 minutes at room temperature. The viral RNA was separated from the coat protein using a linear 7.5-15% sucrose gradient (Brakke and Van Pelt 1970), which was centrifuged at 22,400 rpm in a Beckman SW41 rotor for 16 hours at 4°C. Fractions of 800 μl were recovered and the absorbance of each fraction was measured at 260 nm. RNA was precipitated from the sucrose of appropriate fractions by ethanol precipitation. The resulting pellet was dried and resuspended in double distilled water (ddH_2O) to a concentration of approximately one mg ml^{-1}. Small aliquots were stored at -80°C.

An alternative procedure used for isolating the RNA was by phenol/chloroform extraction. The freshly purified virions were treated with an equal volume of phenol/chloroform to separate

the coat protein from the RNA. The RNA in the aqueous phase was collected by ethanol precipitation, dried and stored in small aliquots as before.

After both procedures a 2 μg sample was reserved for analysis by agarose gel electrophoresis to determine the length and amount of degradation of the isolated RNA .

2.2.5 Preparation of Complementary DNA (cDNA)

The procedure follows the method of D'Alessio *et al.* (1987).

First strand synthesis

A reverse transcription reaction, with a total volume of 50 μl, contained 10 μg PSbMV RNA, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM dithioerythritol (DTE), 3 mM $MgCl_2$, 500 μM of each dATP, dCTP, dGTP and dTTP, 2.5 μg oligo $(dT)_{12\text{-}18}$ primer and 1,000-2,000 units of Murine Moloney Leukemia Virus (M-MLV) reverse transcriptase (BRL). To measure the efficiency of the reaction, a 10 μl aliquot was removed and added to a second tube containing 5.8 μCi of [methyl ^{3}H]-TTP. One μl of this was removed immediately, TCA precipitated and counted to determine the amount of product at 't_o'. Subsequently both tubes were incubated at 37°C for one hour. The reaction was stopped by placing the tubes on ice. At this stage, a second one μl aliquot was removed from the tube containing ^{3}H-TTP, TCA precipitated and counted to determine the amount of product at 't_{60}'.

Second strand synthesis

For the second strand synthesis, the first strand unlabelled reaction was diluted to 320 μl and adjusted to contain final concentrations of 25 mM Tris-HCl pH 8.3, 100 mM KCl, 5 mM DTE, 5 mM $MgCl_2$, 250 μM each of dATP, dGTP, dCTP, dTTP, 500 cpm/pmol [methyl ^{3}H]-TTP, 250 U ml^{-1} *Escherichia coli* (*E. coli*) DNA polymerase I and 8.5 U ml^{-1} *E. coli* ribonuclease H. The reaction was incubated at 16°C for two hours and stopped by the addition of 10 μl 0.5 M EDTA. The yield was determined by the removal of a 5 μl aliquot at 't_o'and 't_{120}' and TCA precipitated as before. The remaining sample was extracted with an equal volume of phenol:chloroform:isoamyl alcohol and the cDNA recovered by precipitation from 70% ethanol and 2.5 M ammonium acetate (Crousse and Amorese 1987). The ethanol precipitation was repeated to remove traces of unincorporated nucleotides, the final pellet being resuspended in 26 μl TE, pH 8.0, (10 mM Tris-HCl, pH 8.0, 1mM EDTA) and stored at 4°C.

The size of the double-stranded (ds) cDNA was determined by agarose gel electrophoresis.

Size selection of cDNA

The ds cDNA was subjected to size fractionation in a 6% polyacrylamide gel (8 ml 30% acrylamide/bis (29:1), 4 ml 10 x TBE, 0.12% ammonium persulphate (APS) and 15 μl TEMED for a 40 ml final volume). The gel was run until the bromophenol blue dye ran off the bottom of the gel. The gel was then lightly stained with 10 μg ml^{-1} ethidium bromide and examined under long wave ultraviolet light to avoid nicking the cDNA. A gel slice containing cDNA greater than 1,600 bp was excised from the gel and placed in an eppendorf tube where it was crushed with a spatula. An equal volume of elution buffer (0.5 M NH_4OAc and 1 mM EDTA) was added. The mixture was vortexed and incubated overnight at 37°C. The supernatant was collected following centrifugation at 13,000 g for 5 minutes in a microfuge. The acrylamide pellet was checked under ultraviolet light for any remaining cDNA. The acrylamide pellet was re-extracted with an equal volume of elution buffer, followed by a two hour incubation at 37°C and spun as above. The procedure was repeated until all cDNA had been removed. The supernatants were combined and the cDNA precipitated with ethanol, dried and resuspended in TE at a concentration of 10 μg ml^{-1}.

2.2.6 Preparation of Vector for Ligation

The plasmid pUC19 was digested with *Sma*I (Promega) at its single recognition site to produce a linear, blunt-ended molecule. 10 μg pUC19 was digested in a 100 μl reaction containing 20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 50 mM KCl and 5 μl *Sma*I (100 U). The reaction was incubated at 25°C for one hour. Following phenol/chloroform extraction, the plasmid was ethanol precipitated, dried and resuspended in 10 mM Tris-HCl pH 8.0.

Calf intestinal phosphatase (CIP) (Boehringer Mannheim) was used to remove 5' phosphate groups in a 50 μl reaction containing 10 μg *Sma*I digested pUC19, 50 mM Tris-HCl pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine and 0.1 U CIP. The reaction was incubated at 37°C for 30 minutes, when a second aliquot of CIP was added followed by a further 30 minute incubation at 37°C. The reaction was diluted to 100 μl with STE pH 8.0 and 0.5% SDS. The reaction was heated to 65°C for 15 minutes, phenol/chloroform extracted, and the plasmid DNA was precipitated with ethanol, dried and resuspended in 10 μl TE, pH 7.5.

2.2.7 Ligation of cDNA into the Vector

The ligation was carried out using two different vector to insert ratios, 4:1 and 2:1. 10 μl ligation reactions were set up containing 5 x ligase buffer (BRL), 1 Weiss unit of T_4 DNA ligase (BRL), and either 80 ng pUC19 and 20 ng cDNA or 80 ng pUC 19 and 40 ng cDNA. The reactions were incubated overnight at 16°C. Two control reactions were set up - one to determine the efficiency of dephosphorylation as indicated by the absence of recircularised pUC19 vector, and the other to test the efficiency of ligation. The reactions were identical to the above except that the former

control tube contained no cDNA and the latter contained 80 ng pUC19 and 20 ng *Rsa*I (BRL) digested plasmid, which had previously been shown to have a high ligation efficiency.

2.2.8 Transformation of *Escherichia coli* JM83

Strain JM83 of *E. coli* was made competent and transformed with the cDNA/pUC19 ligations.

A single colony of JM83 was inoculated into a flask containing 10 ml of Luria-Bertani broth (LB) (1% tryptone, 0.5% yeast extract and 1% NaCl) and incubated overnight at 37°C with shaking. 100 ml of LB were inoculated with 2 ml of the overnight culture and incubated with vigorous shaking until the A_{550} was approximately 0.5. The cells were collected by centrifugation at 500 g for 10 minutes at 4°C. The pellets were gently resuspended in half the original volume with ice cold 50 mM $CaCl_2$ and allowed to stand on ice for 30 minutes. This was followed by centrifugation at 500 g for 10 minutes and the pellets were gently resuspended in 8 mls 50 mM $CaCl_2$. The resuspended cells were stored at 4°C and used within one week of preparation.

200 μl of competent cells was added to each ligation reaction. A control transformation, to test the efficiency of transformation, contained uncut pUC19. The tubes were incubated on ice for 45 minutes. The cells were heat-shocked at 42°C for two minutes, and briefly held on ice before transferring the contents to tubes containing 2 ml LB. The cells were elaborated by incubation at 37°C for one hour. The cells were then pelleted and resuspended in 400 μl LB. Two LB agar plates containing 50 μg ml^{-1} ampicillin and 0.1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) were then spread with 200 μl of the transformed cell suspension and incubated at 37°C overnight to establish colonies. An additional control plate containing competent cells without ampicillin was included to test the viability of the competent cells.

2.2.9 Mini Plasmid Preparation

A rapid alkaline extraction procedure, modified from the method of Birnboim and Doly (1979), was used to screen 80 of the 360 colonies that were unable to metabolise lactose (lac^-) and that were resistant to ampicillin (amp^+). A single colony was dispersed into 3 ml LB and incubated overnight at 37°C with shaking. The cells were collected by centrifugation at 10,000 rpm for two minutes. The pellets were gently resuspended in a solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 7.5, and 2 mg ml^{-1} lysozyme (added just prior to use). After a 10 minute incubation on ice, 400 μl of lysis solution (1% SDS and 0.2 M NaOH) was added, mixed by inverting the tube, and incubated on ice for 5 minutes. 300 μl of acidifying buffer (3 M NaOAc pH 4.8) was then added, mixed by inverting the tube and incubated on ice for one hour. The insoluble material was pelleted by centrifugation at 10,000 rpm for 5 minutes, and the supernatant was decanted into a new tube. The plasmid DNA was recovered from the supernatant by the addition of twice the original volume of 100% ethanol. The precipitate was collected immediately by centrifugation at 10,000 rpm for 5 minutes. The resulting pellet was briefly dried and

resuspended in 300 μl of neutralization buffer (100 mM NaOAc and 50 mM Tris-HCl pH 8.0). After incubating on ice for 2-3 minutes, 700 μl of 100% ethanol was added and the DNA was collected by centrifugation as before. The pellet was dried and resuspended in 40 μl TE.

Aliquots of 3-5 μl were analysed on agarose gels to ascertain the approximate size of the plasmids. Those that appeared to be a reasonable size were subjected to further analysis by digesting with *Eco*RI and *Bam*HI. These restriction endonucleases have restriction sites on either side of the *Sma*I site used for ligation, with only a minimal amount of vector DNA included with the insert. 20 μl digestion reactions containing 10 U *BamH*I (BRL) and 20 U *EcoR*I (NEB), 4 μl sample and 2 μl 10 x React 3 buffer (BRL) were set up. The samples were incubated at 37°C for 1.5 hours then analysed on a 1% agarose gel.

2.2.10 Preparation of Templates for Sequencing

Isolation of RsaI Fragments

Four selected clones (pPSb66, pPSb54, pPSb401 and pPSb402) containing internal *BamH*I or *EcoR*I digestion sites within the insert DNA were prepared for DNA sequencing. Plasmids were purified by the alkaline mini-preparation procedure already discussed (section 2.2.9), then digested in a 100 μl reaction containing 10 μg pUC vector, 30 U *Rsa*I (Boehringer Mannheim) and 10 μl 10 x React 1 buffer (BRL). In addition, 2 μg pUC19 was digested with 10 U *Rsa*I and 2 μl 10 x React 1 buffer in a 20 μl reaction.

The samples were electrophoresed in a 6% polyacrylamide gel and treated as in section 2.2.5 except that two or 3 fragments were collected from each *Rsa*I digested clone. The final pellets were resuspended in 20 μl dH_2O. 3 μl of each isolated fragment was then run on a 1% agarose gel and the amount of cDNA present was estimated by comparison with the intensity of the bands produced by ds DNA markers [1 kilobase (1 kb) markers (BRL)].

Preparation of M13mp18 vector for ligation

The vector M13mp18 was digested with *Sma*I to produce blunt ends suitable for ligation with the *Rsa*I fragments prepared above. A 30 μl reaction containing 5 μg M13mp18, 40 U *Sma*I (Promega) and 3 μl 10 x React 4 buffer was set up. This was incubated for one hour at 25°C, phenol extracted and ethanol precipitated. The pellet was resuspended in 45 μl dH_2O in preparation for dephosphorylation by calf intestinal phosphatase. The reaction was set up as described in section 2.2.6 with the final pellet being resuspended to give a final vector concentration of approximately 20 ng μl^{-1}.

Ligation of the RsaI fragments into the vector M13mp18.

To a 10 μl reaction, 40 ng of *Sma*I cut, phosphatase treated vector, 10-100 ng of *Rsa*I cut fragment, 1 Weiss unit T_4 DNA ligase (BRL) and 2 μl 5 x ligase buffer (BRL) were added. Two control reactions were included. The first, to test the efficiency of the vector dephosphorylation, contained prepared vector but no fragment. The second was dephosphorylated vector with *Rsa*I cut plasmid DNA which had previously been shown to have a high ligation efficiency. All reactions were incubated overnight at room temperature.

2.2.11 Transformation of *E. coli* TG1 cells

E.coli TG1 cells were made competent by the method described in section 2.2.8 for *E.coli* JM83 except that the cells were grown in 2 x YT broth (1% tryptone, 1% yeast extract and 0.5% NaCl). 200 μl of competent cells and 10 μl of ligation mixture were added to sterile test tubes. A control tube containing 200 μl of competent cells and 10 ng of covalently closed circular M13mp18 vector was included to test the efficiency of transformation. The reactions were incubated on ice for 45 minutes, after which the cells were heat-shocked at 42°C for two minutes. The transformed cells were immediately added to a second test tube held at 45°C containing 3.0 ml top agar (1% tryptone, 0.8% NaCl and 0.8% agar), 100 μl of 2% Xgal, 100 μl of 2.5% isopropylthiolgalactoside (IPTG) and 100 μl of untransformed TGI cells. The tubes were gently mixed and immediately poured onto TYE plates (1.0 litre of 2 x YT and 15% agar) and incubated overnight at 37°C. A control plate containing 200 μl of competent cells was included to test the growth of these cells.

2.2.12 Template Preparations (Bankier *et al.* 1987)

Colourless plaques were chosen for template preparation. A single colony of *E.coli* TGI was inoculated into 10 ml 2 x YT and grown overnight at 37°C with shaking. This overnight culture was then diluted 1:100 with 2 x YT to give sufficient volume for 1.5 ml of medium for each plaque.

A single plaque was transferred into a test tube containing the 1.5 ml of diluted TGI cells and shaken vigorously for 5.5 hours at 37°C. Following this, each sample was transferred to an eppendorf tube and the cells pelleted by centrifugation at 13,000 g for 5 minutes. The supernatant containing the phage particles was then added to a second eppendorf tube containing 200 μl PEG solution (20% PEG, MW 6000 and 14.6% NaCl). This was vortexed, then incubated at room temperature for 15 minutes. The phage precipitate was collected by centrifugation at 13,000 rpm for 5 minutes, then the supernatant was removed. The PEG pellet was respun briefly to facilitate removal of all the supernatant before being resuspended in 100 μl TE pH 8.0. An equal volume of phenol saturated with buffer was added to the resuspended PEG pellet, vortexed and centrifuged for two minutes to separate the phases. The aqueous phase was removed and the phage DNA ethanol precipitated. The resultant pellet was washed with 1 ml

100% ethanol, spun and briefly dried *in vacuo*. The pellet was resuspended in 20 μl TE pH 8.0 and stored at -20°C.

2.2.13 Nucleotide Sequence Determination

Sequencing gels were poured according to the method of Sanger and Coulson (1978).

T tracking and sequencing were set up according to Sanger's method of dideoxy DNA sequencing (Sanger et al. 1977) and reactions were set up as described in Bankier *et al.* (1987). The termination mixes comprised:

T termination mix (d/ddT). 3.3 μM dTTP, 36.3 μM dGTP, 36.3 μM dCTP 0.25 mM ddTTP and 1.4 μCi [^{32}P]-dATP. (>400 Ci/mmol specific activity)

G termination mix (d/ddG). 1.7 μM dGTP, 36.3 μM dTTP, 36.3 μM dCTP, 0.1 mM ddGTP and 1.4 μCi [^{32}P]-dATP.

C termination mix (d/ddC) 1.7 μM dCTP, 36.3 μM dTTP, 36.3 μM dGTP, 0.04 mM ddCTP and 1.4 μCi [^{32}P]-dATP.

A termination mix (d/ddATP). 25.6 μM dTTP, 25.6 μM dGTP, 25.6 μM dCTP, 0.01 mM ddATP and 1.4 μCi [^{32}P]-dATP.

Two fragments from clone pPSb54 and one fragment from each of clones pPSb401 and clone pPSb66 were sequenced. The amino acid sequence was inferred from the nucleotide sequence using the DM program (Mount and Conrad 1986).

2.3 RESULTS

Peas infected with the pathotype P1 (Alconero *et al.* 1986) isolate of PSbMV showed the downward curling of the leaves characteristic of infection with PSbMV (Fig.2.1). Some leaves also showed mosaic symptoms, which usually occur early in the infection process.

2.3.1 Virus Purification

Three potyvirus purification procedures were tested for final PSbMV yield. Only the method of Reddick and Barnett (1983) produced more than 1 mg of purified virus from 1 kg of plant material. The method of Reddick and Barnett was extensively modified to increase the yield of PSbMV resulting in high yields of virus (35 mg kg^{-1} plant material) as estimated spectrophotometrically using the extinction coefficient of 2.5 at 260nm for 1 mg ml^{-1} in a 1 cm light path (Hampton and Mink 1975). The use of PEG to concentrate the virus from the initial homogenate and of a Cs_2SO_4 gradient to finally purify the virus resulted in a well-separated opalescent band in the Cs_2SO_4 gradient (Fig.2.2). Purified virus was electrophoresed on an SDS polyacrylamide gel (Laemmli 1970) and was silver stained (Switzer *et al.* 1979). The gel showed a single band with a molecular weight of approximately 33 kDa by comparison with the protein

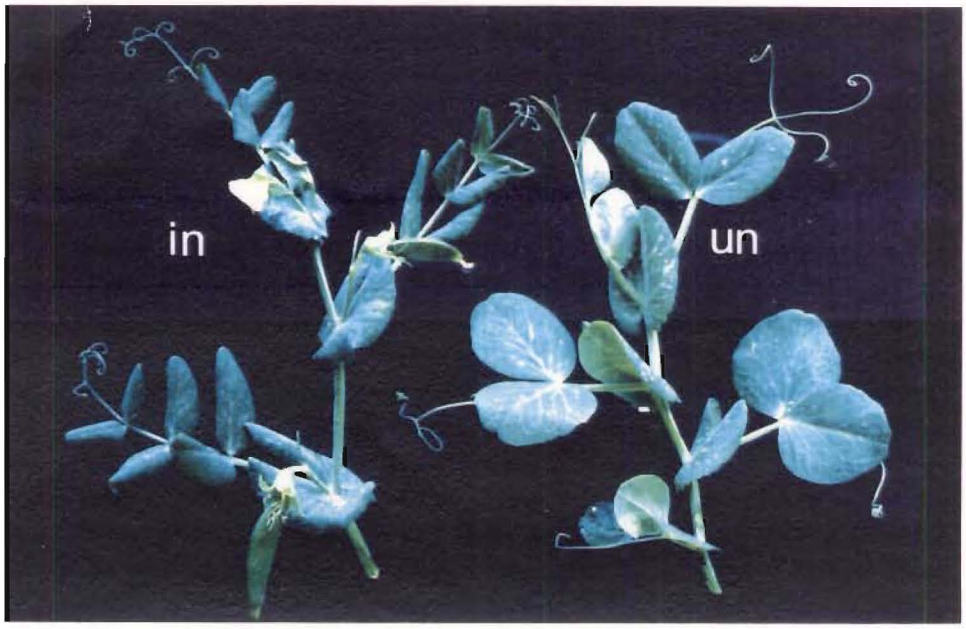


Fig.2.1. The appearance of *Pisum sativum* var. Combie with the severe leaf roll symptoms characteristic of PSbMV infection compared to a healthy plant. in = infected plant. un = uninfected plant.

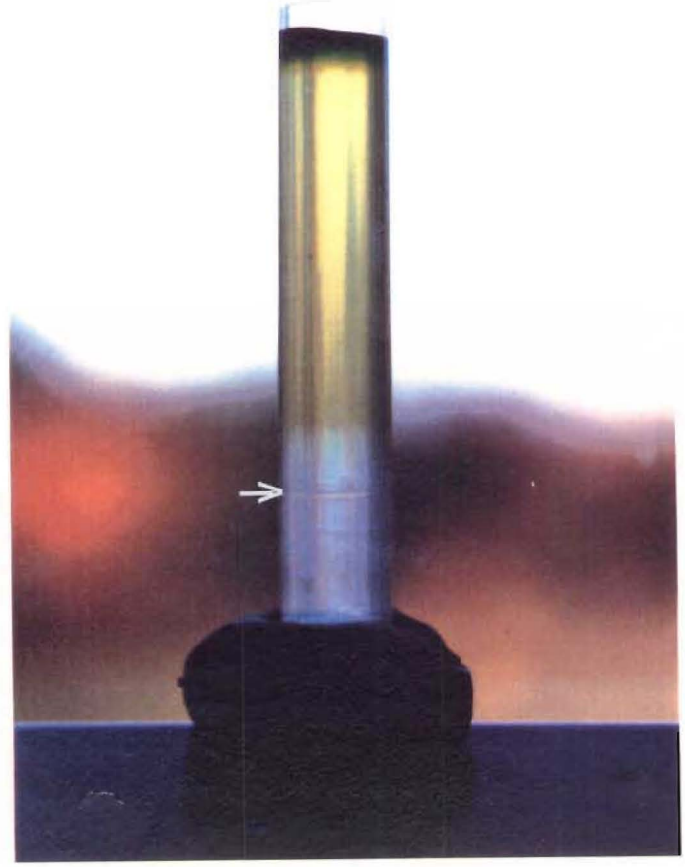


Fig.2.2. A caesium sulphate gradient following ultracentrifugation showing an opalescent virus band (arrowed).

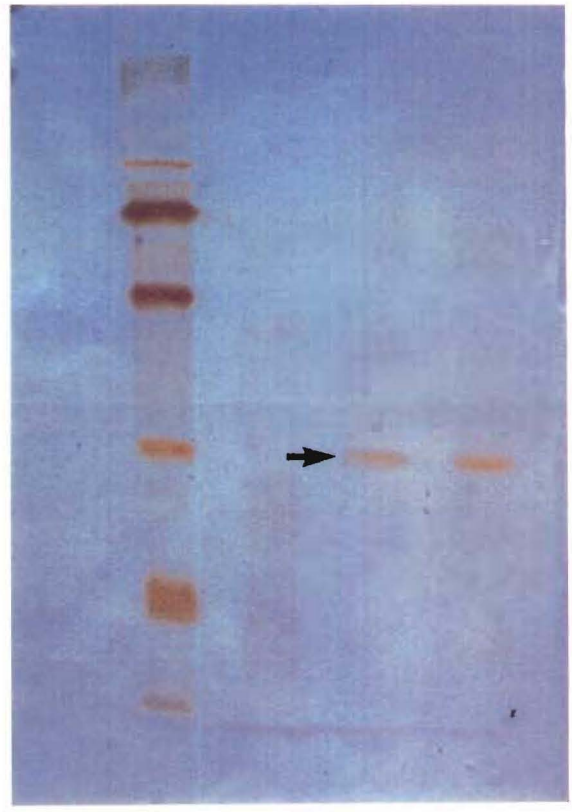


Fig.2.3. A silver stained 10% polyacrylamide gel showing the presence of a single coat protein band with a molecular weight of approximately 33 kDa. Molecular weight markers (Biorad) are as follows: rabbit muscle phosphorylase b (97,400), BSA (66,200), hen white ovalbumin (42,699), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000) and hen egg white lysozyme (14,400).

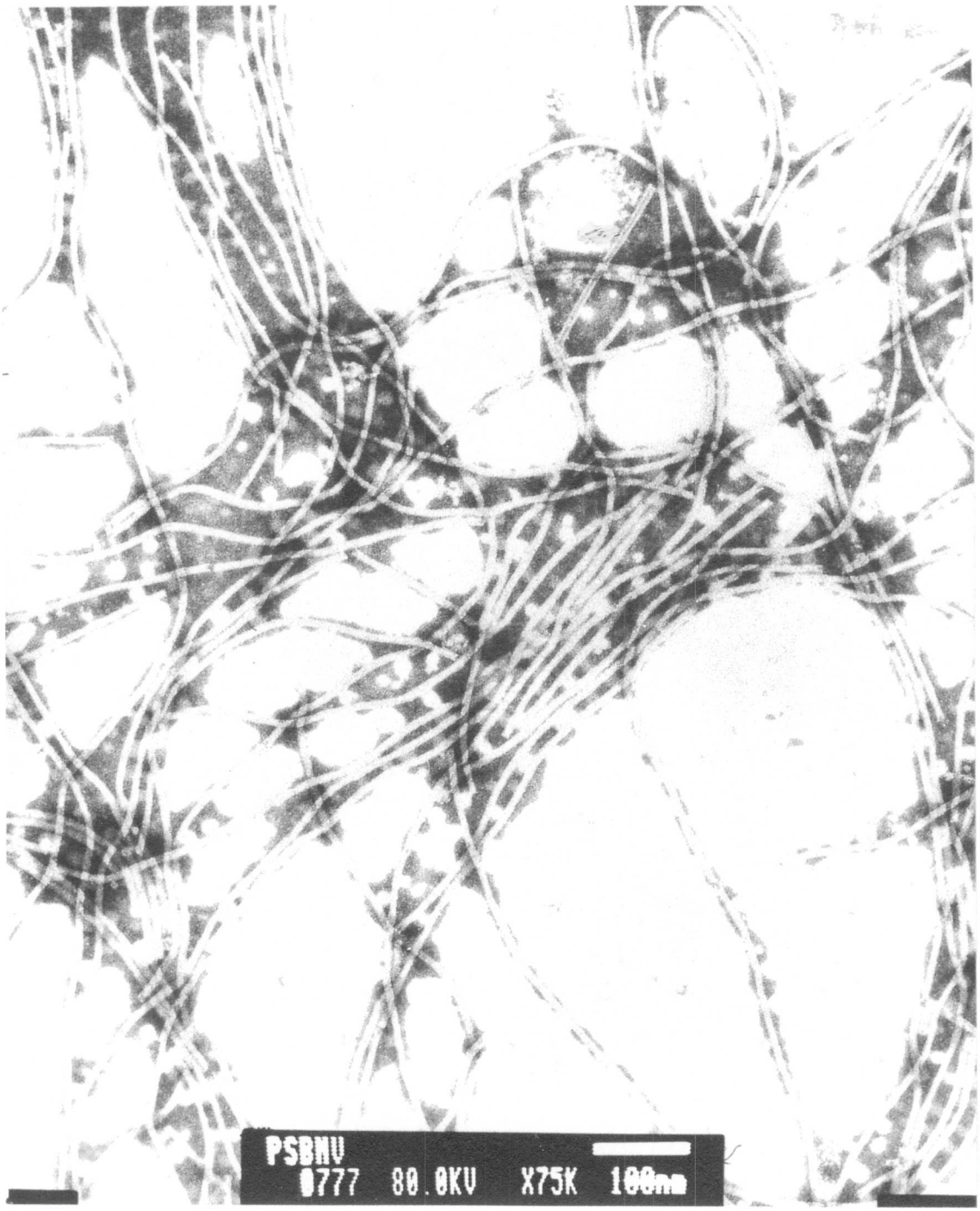


Fig.2.4. PSbMV particles purified by a modified method of Reddick and Barnett. Most particles were intact, with a modal length of 780 nm.

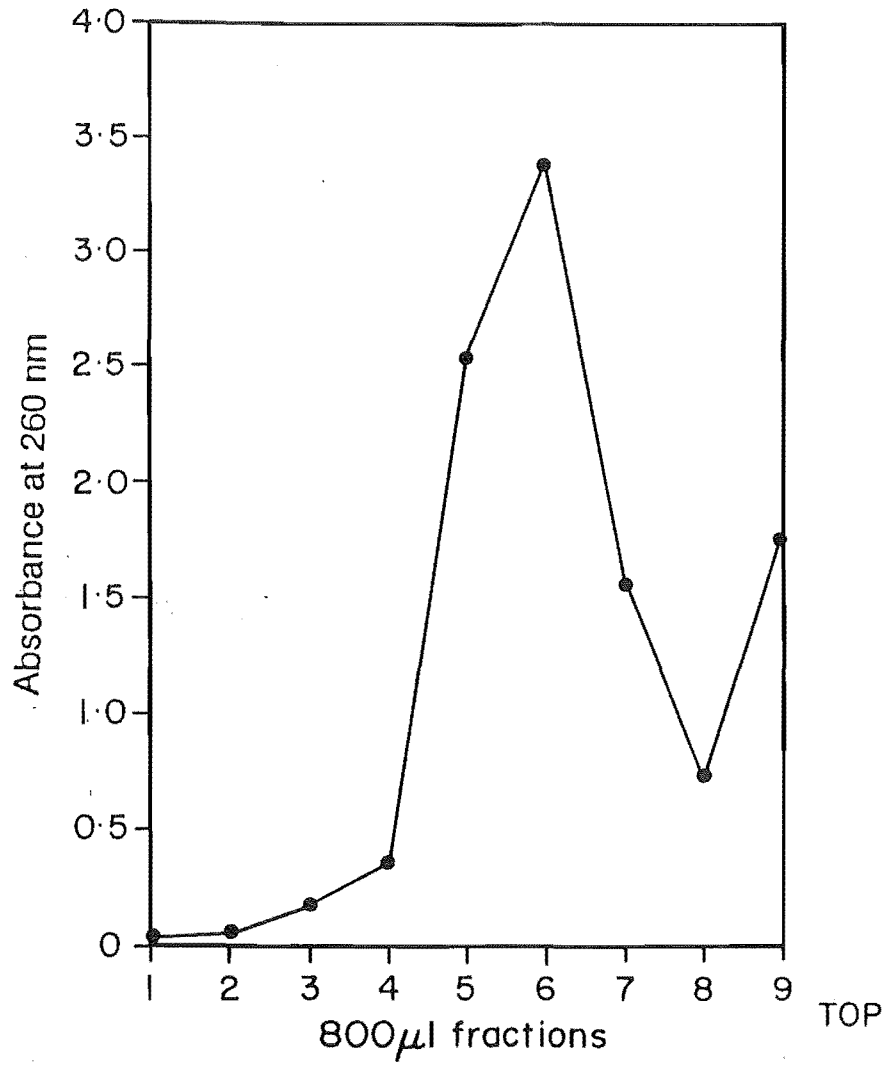


Fig.2.5. Absorbance at 260 nm of fractions containing RNA collected from a sucrose gradient.

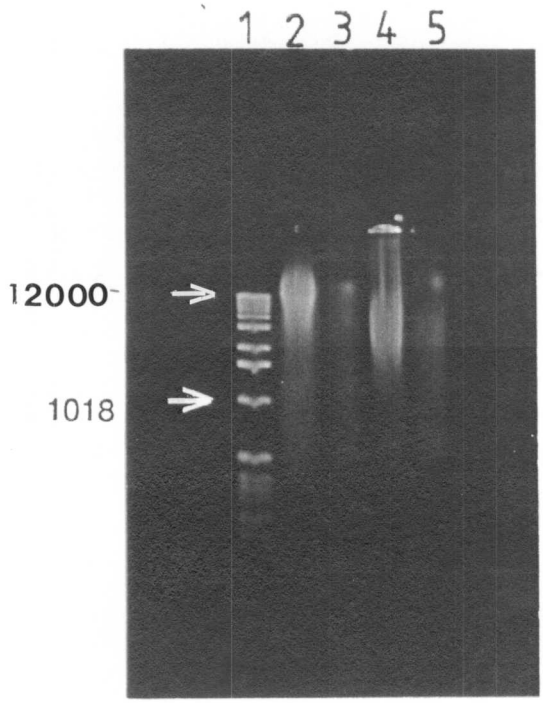


Fig.2.6. Electrophoresis of PSbMV-RNA in a 1% agarose gel. **Lane 1.** 1 kb ladder (BRL). Lanes 2-5. Aliquotes (1 μl) collected from the sucrose gradient shown in Fig.2.5. **Lane 2.** Fraction 6. **Lane 3.** Fraction 7. **Lane 4.** Fraction 8. **Lane 5.** Fractions 5 and 9.

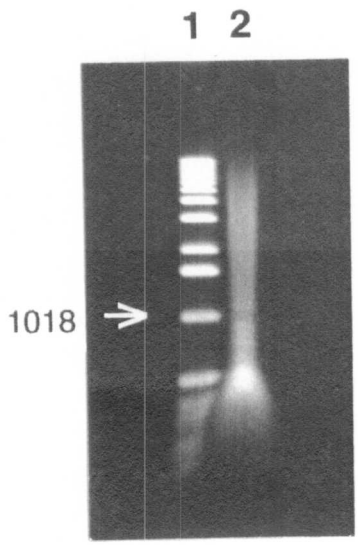


Fig.2.7. Double stranded cDNA made to PSbMV RNA. **Lane 1.** 1 Kb ladder **Lane 2.** 1 μg cDNA.

markers (Fig.2.3). Examination of purified virus stained with PTA using a transmission electron microscope showed virions that were mainly intact and had a modal length of 780 nm (Fig.2.4). To determine the modal length of the virions, 213 particles were scored. The mean length was 797 nm, SD = 75. A hollow particle (as seen in Fig.2.4) is occasionally found. This may be a contaminating virus or an aggregation of cellular material (J.W. Ashby, pers.comm.).

An attempt was made to determine the amino terminal sequence of the PSbMV coat protein by Edman degradation. The sequence reported here is however representative of only 6.5% of the total protein present (as estimated using data on the amino acid alanine in cycle 7). Because the sequence is representative of only 6.5% of the total protein present these data would not appear to represent the N-terminus of the coat protein. The N-terminus may be blocked by an acetylated amino acid. The sequence determined by Edman degradation is ? D ? D V D A G S ? G ? I ? V P. The '?'s denote indeterminable amino acids.

2.3.2 RNA Isolation

The method of Brakke and van Pelt (1970) produced yields of 360 μg RNA from 35 mg of virus purified from 1.0 kg plant material. The full length RNA (approximately 10,000 bp) occurred predominantly in a single 800 μl fraction as shown by the RNA absorbance profile at 260 nm (Fig.2.5). The phenol/chloroform isolation method was successful but yields were lower (approximately 120 μg RNA per 35 mg of purified virus). Agarose gel electrophoresis analysis of RNA prepared by phenol extraction or by sucrose gradients revealed no differences in the quality of the RNA produced by the two methods, as both had some breakage and/or degradation of the RNA (data not presented). However, most of the RNA, as estimated by comparison with ds DNA marker (the 1 kb ladder BRL), appeared to be approximately 10 kb in length (Fig.2.6) Because the higher yields were obtained using digestion with proteinase K followed by a sucrose gradient, this was the method employed to isolate viral RNA.

2.3.3 Production of cDNA

The cDNA produced had a size distribution from approximately 300 bp-8 kb (Fig.2.7). The first strand synthesis was oligo-(dT)-dependent with no first strand being produced in the absence of the oligo-(dT) primer (data not presented).

2.3.4 Ligation into pUC19 and Transformation of JM83

360 colonies that were lac^- and amp^+ were produced from the ligation and transformation events. There appeared to be no significant difference between the numbers of colonies produced from the two different vector:insert ratios. The ratio of 4:1 (vector:insert) produced 169 lac^- colonies while the 2:1 ratio resulted in 191 lac^- colonies. Dephosphorylation of the *Sma*I

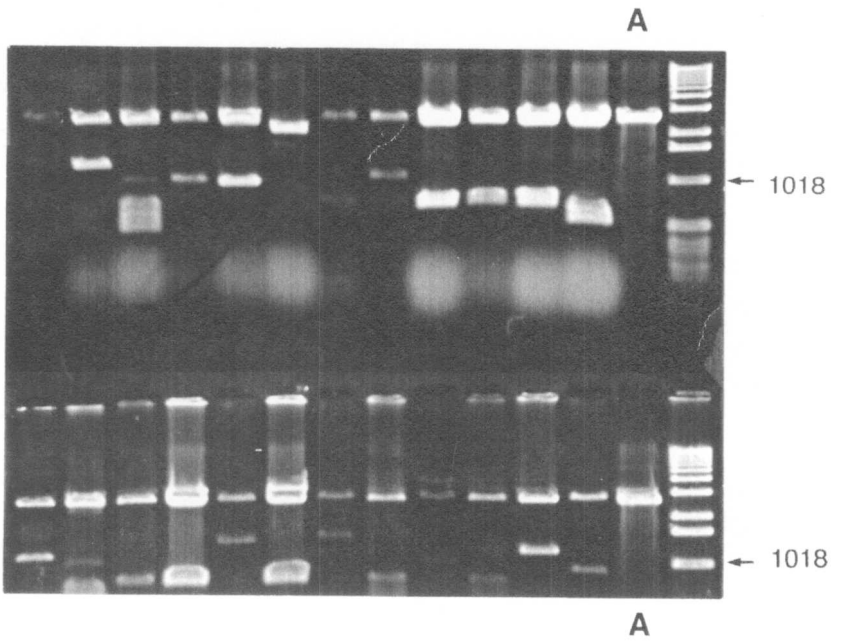


Fig.2.8. Electrophoresis of PSbMV clones digested with *EcoR*I and *BamH*I in a 1% agarose gel. The 1 Kb ladder (BRL) was used as markers **Lane A.** *EcoR*I digested pUC19.

Table 2.1 Summary of cDNA clones of which some of the *Rsa*I fragments were sequenced.

Clones	Internal BamHI or EcoRI restriction sites		Cloned in M13mp18	Sequenced
pPSb 60	+	*	2 RsaI fragments	1 RsaI frament†
pPSb 54	+	*	2 RsaI fragments	2 RsaI fragments†
ppSb 401	+	*	1 RsaI fragment	1 RsaI fragment†
pPSb 402	+	*	-	-
pPSb 70	-	*	See Appendix A	See Appendix A
pPSb 13	-	*	See Appendix A	See Appendix A
pPSb 67	-	*	See Appendix A	See Appendix A
pPSb 1A	+	*	-	-
pPSb P	-	*	-	-
pPSb 29	-	*	-	-

* see Fig. 2.9
† see Fig. 2.11

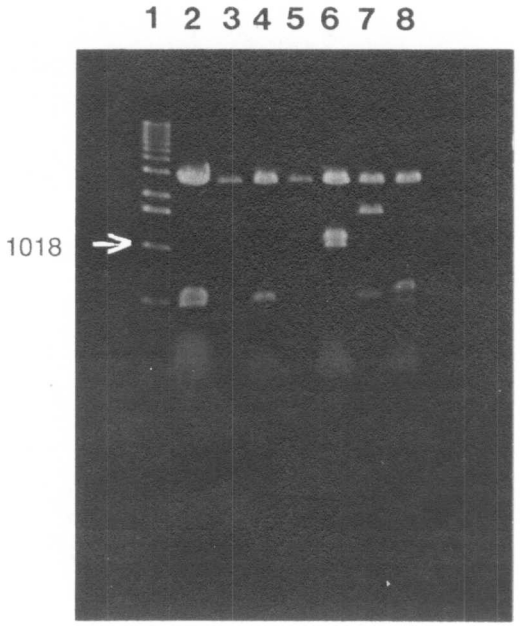


Fig.2.9. Electrophoresis in a 1% agarose gel of plasmids containing cDNAs with internal *EcoR*I and/or *BamH*I restriction sites . **Lane 1**. 1 kb ladder (BRL). **Lane 2.** Clone pPSb1A. **Lane 3.** Clone pPSb54. **Lane 4.** Clone pPSbP. **Lane 5.** Clone pPSb29, **Lane 6**. Clone pPSb402. **Lane 7.** Clone pPSb401. **Lane 8.** Clone pPSb66.

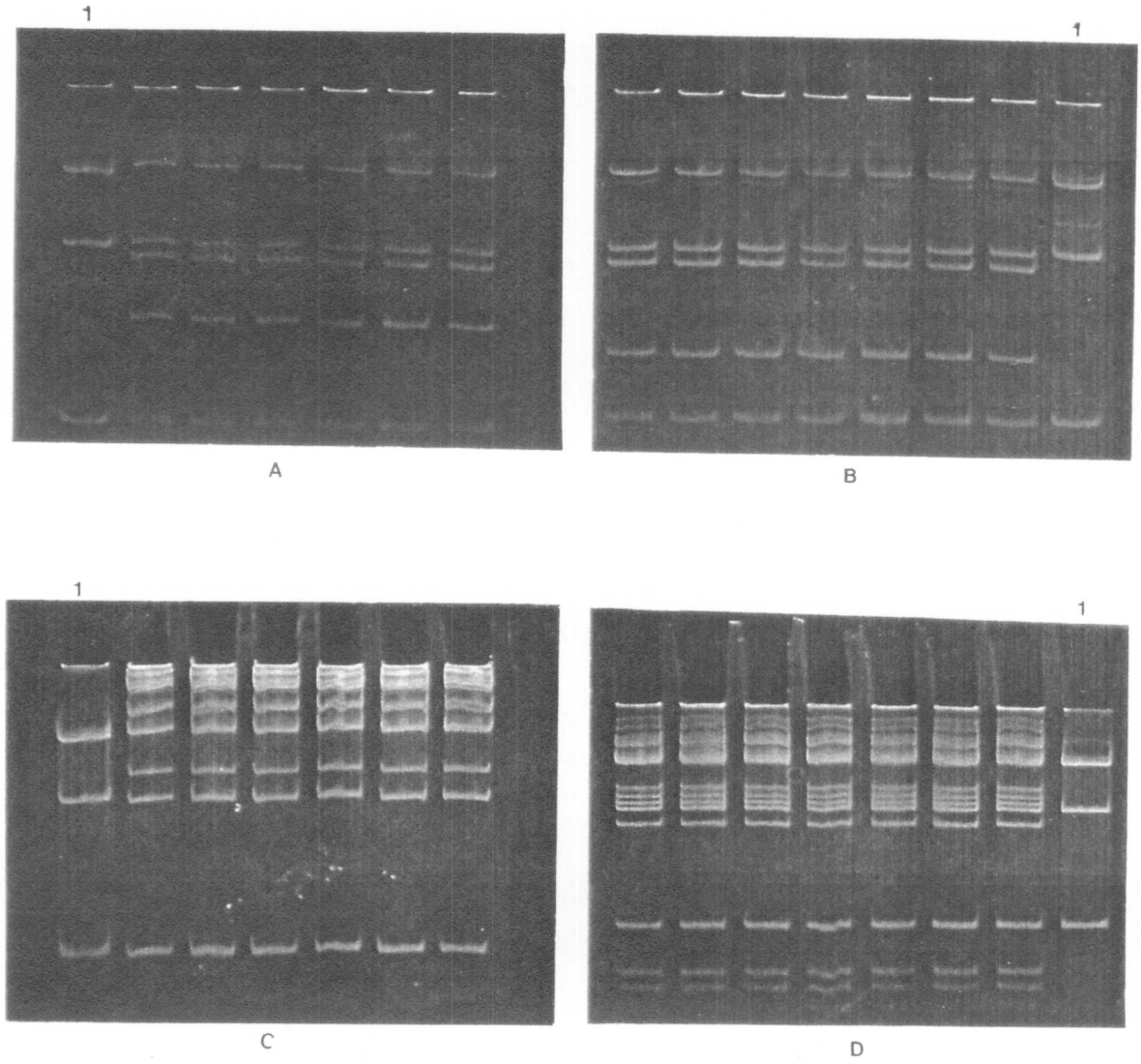


Fig.2.10. Preparative polyacrylamide gel electrophoresis of *Rsa*I digested clones **A**. pPSb54, **B**. pPSb66, **C**. pPSb401 and **D**. pPSb402. **Lane 1.** *Rsa*I digested pUC19.

digested pUC19 was efficient in preventing recircularisation of the plasmid with only 12 colonies (all lac^+) growing on the control plate.

2.3.5 Analysis of the Inserts

Clones from the cDNA library were analysed to determine the size of the inserts they contained and whether or not they contained restriction sites for either *Bam*HI or *Eco*RI. The restriction endonucleases *BamH*I and *EcoR*I were chosen because they have restriction sites on either side of the *Sma*I site used for ligation, with only a minimal amount of vector DNA included with the insert. Three clones (pPSb70, pPSb13, and pPSb67) had been previously characterised by sequence analysis (Appendix A). These clones were shown to contain coat protein coding sequences, but none contained restriction sites for either *Bam*HI or *Eco*RI restriction endonucleases. Several clones in the cDNA library, however, had restriction sites for either *Bam*HI or *Eco*RI. Four of these clones (pPSb401, pPSb402, pPSb66, and pPSb54) were further characterised in the hope that they may encode non-coat protein sequences.

Plasmid DNA prepared from eighty of the 360 lac^- amp^+ colonies was digested with *BamH*I and *EcoR*I. All contained inserts which ranged in size from approximately 300-2000 bp, estimated by comparison with the 1 kb ladder. A representative gel showing 24 such digests is shown in Fig.2.8. Five of the inserts contained internal *BamH*I or *EcoR*I restriction sites, giving three bands, one of which is pUC19, on the agarose gels (Fig.2.9). The clones were: pPSb1A (lane 2), pPSb54 (lane 3), pPSb402 (lane 6), pPSb401 (lane 7) and pPSb66 (lane 8). The other two clones that appear in Fig.2.9 (lanes 4 and 5) did not contain internal restriction sites for these two restriction endonucleases. Table 2.1 summarises the analysis of the various clones.

Four clones which have internal *BamH*I and *EcoR*I restriction sites were digested with the restriction endonuclease *Rsa*I (Fig. 2.10). The results indicate that 3 of the clones (pPSb401, pPSb402 and pPSb66) were unrelated to each other while one, pPSb54, shared at least some sequences with pPSb66 (Fig.2.10). pUC19 digested with *Rsa*I was used to identify the pUC19 bands in the digested clones.

2.3.6 Ligation of *Rsa*I Fragments into M13mp18 and Transformation of *E. coli* TGI

The ligation of fragments isolated from the *Rsa*I digested clones pPSb401, pPSb402, pPSb66 and pPSb54 and subsequent ligation into the cloning vector M13mp18 was achieved for 5 fragments from 3 clones (see Table 2.1). These were two fragments from clones pPSb54 and pPSb66 and one fragment from clone pPSb401. Fragments from clone pPSb402 did not ligate into M13mp18. Work on this clone was discontinued in this study. *E.coli* strain TGI was transformed, and templates were produced for nucleotide sequencing.

Fig. 2.11

Clones pPSb66 fragment I and pPSb54 fragment I.

1	TGG	TGC	TTT	TCC	TTC	CTA	GCT	ATC	TCT
	W	C	F	S	F	L	A	I	S
	TAT	GGT	TCC	GTT	CTA	ACA	ACC	AAT	AAT
	Y	G	S	V	L	T	T	N	N
	AGA	ATT	TCC	GAA	TAT	GCT	GCA	AAA	GCT
	R	I	S	E	Y	A	A	K	A
	CGT	CGT	AAC	CCC	AAG	CTT	102		
	R	R	N	P	K	L	Total 33aa		

Clone pPSb54 fragment II. - complementary strand.

93	TAT	CAT	GTC	ATC	TTA	GGG	GCC	ATC	GAC
	Y	H	V	I	L	G	A	M	D
	CGT	AGG	AAT	TAC	GAA	GTT	TTA	CGC	TEG
	R	R	N	Y	E	V	L	R	W
	GTG	GAA	CGA	ATC	CYA	ATC	AAG	CTA	CTG
	V	E	R	I	L	I	K	L	L
	ACG	GTT	GGC	TCT	1				
	T	V	G	C	Total 31aa				

Clone pPSb401 fragment I.

1	CCG	ACT	CAG	ATT	TCT	ACA	TCA	CCA		
	P	T	Q	I	S	T	S	P		
	GTC	ACT	GCA	AGC	CAG	AGC	ATA	TAT	AAG	CTG
	V	T	A	S	Q	S	I	Y	K	L
	CTG	AAC	AGC	ACT	AGC	GAT	75			
	L	N	S	T	S	E	Total 24aa			

Fig.2.11. The unambiguous nucleotide sequences and inferred amino acid sequences of four *Rsa*I fragments from three clones. The fragment I's from clones pPSb66 and pPSb54 were identical.

2.3.7. Sequence of Clones

The sequence data presented for the *Rsa*I fragments of each of the clones are not complete because the top and bottom of the gel were not able to be read. As a consequence only sequence data from easily read parts of the lanes are presented. The inferred amino acid sequence from these sequence data for fragments from the clones pPSb54 (two fragments), pPSb66 (one fragment), and pPSb401 (one fragment) revealed that each had a single open reading frame. Alternative reading frames all contained at least one stop codon (data not presented). Two fragments, one from clone pPSb54 and one pPSb66, had the same sequence. A total of three fragments were unique; one was derived from pPSb54, one from pPSb401, and the one common to pPSb54 and pPSb66 has already been mention above. The nucleotide sequence data and the inferred amino acid sequence that gave an open reading frame for fragments from each of these clones are shown in Fig.2.11.

2.4 DISCUSSION

The purification of PSbMV proved to be difficult. Initially, broad beans (*Vicia faba*) were used as the host plant in an attempt to avoid using commercial pea seed which may be infected with PSbMV. This would have meant that a single strain of PSbMV could not be guaranteed. However, attempts to purify virus from broad beans were unsuccessful. Difficulty in purifying virus from broad beans has also been experienced by other workers (T.M.A. Wilson pers. comm.). Subsequently, peas inoculated with pathotype P1 of PSbMV (Alconero et al., 1986) were used.

The methods of Hamilton and Nicols (1978) and Knesek *et al.* (1974) both use low molarity extraction buffers. These are less effective than high molarity buffers in preventing adhesion of the virus particles to plant tissues and ineffective in preventing aggregation of the virus particles (Stace-Smith and Tremaine 1970). Neither the procedure of Hamilton and Nicols (1978) nor that of Knesek *et al.* (1974) produced acceptable yields of virus in my hands. The method of Reddick and Barnett (1983) combines high molarity phosphate extraction buffers with PEG precipitation and caesium sulphate density gradients to purify and concentrate the virus. The method also utilises chloroform , which Inouye (1967) observed was the only solvent not to affect PSbMV particles adversely, to clarify the extract. My experience with the virus indicated that PSbMV is sensitive to urea. As a consequence, this was eliminated from the extraction buffer and reduced to half the original concentration in the buffer used to resuspend the first PEG pellet. β-mercaptoethanol and ascorbic acid were added to the extraction buffer as additional protection against the action of oxidising agents endogenous to plant tissue. A further modification to Reddick and Barnett's procedure was to increase the concentration of PEG for the first precipitation event from 4% to 5%. The pellet resulting from concentrating the virus with 4% PEG was very soft and tended to break up.

The modifications gave a high-yielding, reliable purification procedure for PSbMV. The observation of virus particles 780 nm in length following purification is in close agreement with the 770 nm length published for PSbMV by Hampton and Mink (1975). The integrity of the particles is important and allows the isolation of full length RNA. A single band on a silver stained SDS gel confirmed the purity of the virus isolated using this method. The yield of 35 mg of virus for each kg of plant material is similar to the published values for other potyviruses. Reddick and Barnett (1983) reported 30-50 mg kg^{-1} for BYMV and pea mosaic virus. Knesek *et al.* (1974) reported a value of 55 mg PSbMV kg^{-1} plant material, and Hamilton and Nicols (1978) obtained 50 mg kg^{-1} using their procedure.

It was important to isolate RNA from freshly purified virus. Storing the virus following purification resulted in RNA that was degraded. The sucrose gradient method of Brakke and van Pelt (1970) gave relatively high yields of RNA. At least one of the 800 μl fractions taken from the gradient had a high proportion of full length RNA. It is interesting to note that although all glassware and plasticware was rendered RNase free, degradation occurred. Also in isolating RNA, the virus particles were digested with proteinase K to remove the coat protein. This step would also be expected to digest any RNase that had been carried through from the plant material. However, some degradation of the RNA still occured. This may be due to the shearing of the long single-stranded RNA molecule, as mentioned briefly in section 2.1. Although shearing of RNA is not considered to be the problem that it is for DNA, encapsidation of the RNA by coat protein may alter the behaviour of the molecule. An indication that RNA behaves differently when encapsidated is demonstrated by purified potato virus Y virions being non-infectious after storage at -20°C (A.P. Fellows pers. comm.). Therefore, PSbMV RNA may be more suseptible to shearing when encapsidated than when in a purified form. Hampton *et al.* (1974) reported the predisposition of PSbMV to breakage. Indeed, an earlier measurement of the virus reported a bimodal distribution of particle length (Hampton *et al.* 1974). The difficulty in isolating RNA is summed up by Palukaitis (1984) who stated that "the best one (method) is that consistently yielding the most RNA in an intact state *in one's own hands*". This statement is very pertinent.

While the transformation efficiency of JM83 was high, the efficiency of ligation into pUC19 appeared to be poor with 360 colonies resulting from 500 ng cDNA. Analysis of the inserts revealed that none of the 80 clones screened contained inserts of more than 2.4 kbp. This is interesting, considering that the cDNA was subjected to size selection following synthesis, with only cDNA of 1.6 kbp or above being used for ligating into pUC19. The phenomenon has also been reported by T. Turpen (in press). He states that the size of the inserts cloned is significantly smaller than the size of the cDNA synthesised and selected. He offered no explanation for this observation. It is known, however, that size selection on polyacrylamide gels results in some smaller fragments contaminating the larger fragments. As smaller fragments ligate into pUC vectors more efficiently than larger ones, a disproportionate number of clones containing small

inserts can be observed. In addition, smaller plasmids may have a competitive advantage during transformation. The number of clones found to contain inserts of approximately 1.6 kb was also disproportionately high. Reverse transcriptase is known to pause at certain secondary structures of the RNA. It is here that the molecule is most likely to "fall off", resulting in clusters of cDNA being produced with similar lengths.

Two classes of cDNA clones were generated using oligo-(dT) as a primer. The first class encodes the coat protein gene, contain 3' poly(A) tract and do not contain restriction sites for the endonucleases *Bam*HI or *Eco*RI (see Appendix A). The second class are distinguished from the first by containing restriction sites for *Bam*HI and *Eco*RI and thus would not appear to be derived from the 3' end of the genome where the oligo-(dT) would have been expected to have annealed to the poly(A) tract. The dependence on oligo-(dT) primer for the production of first strand cDNA suggests that PSbMV is polyadenylated. Further evidence comes from sequence data analysis of clones pPSb70, pPSb13 and pPSb67 (Appendix A). Other potyviruses including TVMV have been shown to be polyadenylated at the 3' end of the genome (Hari *et al.* 1979; Hellmann *et al.* 1980). Without sequence data for PSbMV, however, less confidence could be placed in the polyadenylation of PSbMV RNA. There is also a second general class of clones in the cDNA library which would appear to be derived from elsewhere in the genome. The second class of clones may have arisen by oligo-(dT) annealing internal 'A'rich sequences. In Appendix A an area from nucleotide 432 to 453 is indicated as one possible areas to which oligo-(dT) may be able to anneal. This region contains 17 'A's with only two 'T's, one 'G', and one 'C' interspersed. Thus the observed dependence on oligo-(dT) for the production of first strand cDNA may have been due to the annealing of this primer to 'A' rich stretches of the genome rather than to the poly[A] tail. An alternative way by which the second class of clones may have arisen is by incomplete second strand synthesis.

An attempt was made to determine the origins of those clones that did not appear to encode the coat protein gene (the second class). Some of the fragments of the clones in question were sequenced and a comparison between the inferred amino acid sequences of TEV and TVMV and the unambiguious amino acid sequences of the *Rsa*I fragments was conducted using the FASTA sequence package (Pearson and Lipman 1988). Comparison of the inferred amino acid sequences for four fragments of these clones revealed that each had a single open reading frame. Two of the four had overlapping sequences. This pair and the remaining two fragments were different. The FASTA sequence package (Pearson and Lipman 1988) revealed no amino acid sequence similarities between the two virus sequences and the clones. The position of these clones in the PSbMV sequence was therefore unable to be established. It is not surprising that regions of similarity between these clones and TEV and TVMV were not observed. The amino acid sequences representing the clones were quite short, being 33, 31, and 24 amino acids long. A low sequence similarity was observed between major regions of the polypeptides of

TEV and TVMV by Domier *et al.* (1987). They investigated the sequence similarity between a number of gene products of TEV and TVMV. The sequence identity between the NIb proteins of these two viruses was 66.9%, but much of this homology was due to two highly conserved regions. The rest of the protein demonstrated very little sequence similarity. The NIa proteins demonstrated only 48.8% identity, again with two clusters of conserved amino acids. These show that most of the sequence is variable, except for the rare highly conserved regions within the various polypeptides.

Edman degradation was used in an attempt to determine the N-terminal amino acid sequence of the coat protein of PSbMV. The results of the chemical sequencing, however, represented only 6.5% of the total protein present. The most probable explanation is that a high proportion of the protein was blocked to Edman degradation and that a degradation product of the coat protein was sequenced. Some potyviruses are known to have blocked N-termini (Shukla and Ward 1988). This would explain why a minority protein was sequenced. Some potyviruses, for example plum pox virus (Lain *et al.* 1988), have been shown to have truncated coat proteins. The truncated capsid protein of plum pox virus is 260 amino acids long while the total coat protein consists of 330 amino acids. The PSbMV coat protein is 287 amino acids long; the position of the amino acids sequenced chemically is 53 amino acids downstream (see boxed region in Appendix A). The location of the sequence ? D ? D V D A G S ? G ? I ? V P coincides with the N-terminus of the trypsin resistant core described by Shukla *et al.* (1988b) for other potyvirus coat proteins. This suggests that the coat protein of PSbMV may undergo degradation similar to that experienced with other potyviruses.

In conclusion, the difficulties in purification of PSbMV and the isolation of the RNA were largely overcome. The resultant RNA was of sufficiently high quality to be used to generate a cDNA library and to be used to programme *in vitro* translation in rabbit reticulocyte lysate to produce viral proteins (Chapter 5) and to programme protoplasts (Chapter 6). The use of oligo-(dT) and ligation into the vector without the use of linkers appears to offer a good strategy for the production of a cDNA library where there is a highly enriched source of a specific RNA, as is the case when dealing with RNA plant viruses.

CHAPTER 3

THE INCLUSIONS INDUCED BY PSBMV

3.1 INTRODUCTION

The types and intracellular locations of inclusion bodies are among the 49 criteria listed for classifying plant viruses into groups (Harrison *et al.* 1971). Members of the potyvirus family demonstrate a variety of inclusion bodies, including cylindrical, nuclear and amorphous inclusions. These have been shown to be aggregations of immunogenically distinct virus-encoded proteins (Hiebert *et al.* 1971; Shepherd *et al.* 1969).

All potyviruses induce cytoplasmic cylindrical inclusions which appear as pinwheels in cross-section. The presence of these inclusions are diagnostic for potyvirus infection (Edwardson 1966, 1974). In addition, the cylindrical inclusions induced by various members of the group are morphologically distinct, causing Edwardson (1974) to suggest that they may povide useful characters to subdivide the group. His classification proposed that viruses in subdivision-I induce cylindrical inclusions containing scrolls and pinwheels, while those in subdivision-II induced laminated aggregates and pinwheels. Subdivision-III members induce pinwheels, scrolls and laminated aggregates, and subdivision-IV members induce pinwheels, scrolls and short curved laminated aggregates (Edwardson *et al.* 1984).

Besides the cylindrical inclusions, some members of the potyvirus family induce additional aggregates in infected host cells. These aggregates occur in either the cytoplasm (amorphous inclusions) or the nucleus (nuclear inclusions) or both. TEV is a notable example producing large (6-8 μm) flat crystals in the nuclei of infected cells (Christie and Edwardson 1977). The nuclear inclusion bodies of TEV are composed of two distinct proteins that aggregate in equimolar amounts (Dougherty and Hiebert 1980b). The proteins are termed NIa and NIb. Although very few members induce amorphous and/or nuclear inclusions, a number of them have been shown to produce the proteins of which the inclusions are composed, for example soybean mosaic virus (SMV) (Vance and Beachy 1984). It has been demonstrated for some potyviruses that the NIa and NIb proteins are present both in *in vivo* and *in vitro* translations, for example TEV (Dougherty and Hiebert 1980b; Hiebert *et al.* 1971).

The amorphous inclusions are also rarely observed in potyvirus-infected cells. Like the proteins composing the nuclear inclusions, the amorphous inclusion protein is encoded by the potyvirus RNA (de Mejia *et al.* 1985a) and is expressed in infected host cells. The amorphous inclusion protein is believed to have two functions, that of helper component [which aids the uptake of virus by the insect vector (Thornbury *et al.* 1985)] and as a putative protease

responsible for some of the cleavages of the polyprotein (Carrington *et al.* 1989). Further discussion of the role of this protein can be found in sections 4.4 and 5.1.

The cytoplasmic inclusions induced by PSbMV are described in this chapter. The morphologies of the cylindrical inclusions observed in this study are used to place PSbMV into subdivision-IV according to the criteria outlined by Edwardson *et al.* (1984). PSbMV had previously been assigned to subdivision-III (Edwardson 1974). After the creation of subdivision-IV from elements of subdivision-III (Edwardson *et al.* 1984), PSbMV remained in subdivision-III. This study also attempts to determine the location of one of the proteins that compose the nuclear inclusion body after preliminary studies provided no evidence for the presence of the nuclear inclusions.

3.2 MATERIALS AND METHODS

Table 3.1 Viruses to which Primary Antisera were made to the suppliers.

Protein	Virus encoding the virus	Abbreviation used	Supplied by
CI	PSbMV	PSbMV CI	D. Gonsalves, Cornell Univ., Ithaca, USA
CP	PSbMV	PSbMV CP	G. Mink, Washington State Univ., Washington, USA
NIa (49k)	TEV	TEV NIa	E. Hiebert, Univ. of Florida, Florida, USA
NIb (54k)	TEV	TEV NIb	E. Hiebert, Univ. of Florida, Florida, USA
AI	WMMV	WMMV AI	E. Hiebert, Univ. of Florida, Florida, USA
AI	PeMV	PeMV AI	E. Hiebert, Univ. of Florida, Florida, USA
CP	MDMV	MDMV CP	D. Teakle, Univ. of Queensland, St Lucia, Australia

3.2.1 Preparation of Samples for Transmission Electron Microscopy

Whole virus

Grids for observing whole virus in infected plant tissue were prepared as outlined in section 2.2.1.

Cylindrical, Amorphous and Nuclear Inclusions

Sections (1 mm) of infected pea leaf and 5 mm lengths of infected pea root were fixed for 2-3 hours in 2.5% glutaraldehyde in 0.075 M Na_2HPO_4, pH 7.2 (PO_4 buffer), and 0.5% caffeine. The samples were washed 3 times for 10 minutes each in 0.075 M PO_4 buffer. Post-fixation involved

2-3 hours in 2% osmium tetroxide in 0.075 M PO_4 buffer, followed by a short wash in PO_4 buffer. Dehydration of the samples was achieved by holding the samples in 20, 40, 60, and 80% acetone for 10 minutes each, followed by 3 changes of 100% acetone of 10 minutes each. The samples were placed in a vial containing 30% Spurr's resin in acetone and rotated for several hours. The lower concentration of resin was replaced by 70% resin and rotated overnight. The following day, the samples were arranged in 100% Spurr's resin and baked at 70°C overnight to polymerise.The samples were cut on an ultramicrotome with a glass knife and mounted on copper grids coated with formvar. The samples were stained with 2% uranyl acetate for 10 minutes, rinsed 3 times in dH_2O and then stained with lead citrate for 2 minutes. The grids were rinsed in dH_2O and examined under a Jeol JEM 1200Ex microscope.

3.2.2 Immunofluorescence

The method for fluorescence follows that outlined by D. Scott, Christchurch Clinical School. Protoplasts were isolated from infected pea leaves as outlined in Chapter 6.2.3. A drop containing the protoplasts was placed on a slide coated with 1% gelatin. The slide was air dried, then immersed in 100% ethanol for 15 minutes. After air drying again, the slide was flooded with 200 μl of 1:500 dilution of primary (1°) antiserum (either PSbMV coat protein or PSbMV cylindrical inclusion antiserum see Table 3.1) in PBS (4mM KH_2PO_4, 16mM Na_2HPO_4, 115mM NaCl; pH 7.4). The slide remained in a humidified chamber for 30 minutes at room temperature, after which it was washed briefly with dH_2O, followed by a 15 minute wash in 0.5 M KH_2PO_4. The slide was drained briefly and flooded with 200 μl of 1:20 dilution of secondary (2°) antibody (donkey-anti-rabbit conjugated to fluorescein isothiocyanate, a gift from D. Scott, Christchurch Clinical School, Christchurch). The slide was left in a humidified chamber for 30 minutes at room temperature, drained and mounted in glycerol/PBS (2:1) pH 8.6. The slide was examined using an Olympus epifluorescent microscope and photographed on Kodacolor (200 ASA).

Immunofluorescence was also carried out in an attempt to localise the NIb protein. The procedure was the same as described above except that the primary antiserum was to TEV NIb.

For all experiments involving immunofluorescence, "blind" experiments were conducted. In this procedure the slides were given code numbers by another person. They were then examined before decoding.

3.2.3 Isolation of fraction enriched in nuclei from infected and uninfected pea material for Western blots

The method of Kodrzycki *et al.*(1989) was used to isolate nuclei from infected and uninfected pea leaves. Pea leaves (0.1 g) were frozen at -80°C for 10 minutes. The frozen material was ground gently in a mortar and pestle with 2 ml of isolation buffer (0.44 M sucrose, 25 mM Tris-HCl pH 7.8, 0.1% (w/v) Triton X-100, 10 mM $MgCl_2$, 2.5% (w/v) Ficoll, 5.0% (w/v) dextran sulphate and 10

mM β-mercaptoethanol). The mixture was filtered through Miracloth and the nuclei pelleted by centrifugation at 1000 g in a bench top microfuge for 5 minutes. The pellet was resuspended in isolation buffer and centrifuged as before. The supernatant was reserved for analysis and the major portion of the pellets was resuspended in 400 μl of PBS, pH 7.4. A small amount of the pellets was reserved for microscopic examination. For this, the nuclei were stained with a drop of carmine red and examined using a light microscope to determine integrity and quality. Samples (10 μl) were prepared for SDS-PAGE and Western blots as outlined in section 4.2.4. The antiserum to the TEV NIb protein was used as the 1° antiserum.

3.2.4 Immunogold labelling (Tomenius et al. 1987)

Sections from infected pea leaves and roots were prepared as described in section 3.2.1 except that the sections were mounted onto gold grids. All grids were immersed in 30 μl drops of the appropriate solution on parafilm and incubated at 25°C in a humidified chamber. The sections were incubated in 1% bovine serum albumin (BSA) in 0.15 M NaCl, 0.01 M Na_2HPO_4 buffer with 0.1% Tween 20 pH 7.0 (PO_4/Tween) for 15 minutes. The grids were then transferred to the appropriate 1° antibody diluted in PO_4/Tween containing 0.01% BSA. The 1° antisera dilutions were: TEV NIb 1:4000, PSbMV CP 1:4000, PSbMV CI 1:3000 and WMMV AI 1:3500.

Sections were incubated with the 1° antiserum for one hour and rinsed with PO_4/Tween, followed by a second rinse of PO_4/Tween plus 0.01% BSA. The rinses were repeated 3 times. The grids were incubated in goat-anti-rabbit antiserum coupled to 5 nm colloidal gold diluted 1:50 with 0.01% BSA in PO_4/Tween for 15 minutes. The grids were then washed as above. The sections were stained with 2% uranyl acetate in water for 10 minutes. The sections were rinsed 3 times in dH_2O and stained with lead citrate for 2 minutes. The sections were rinsed in dH_2O. The positive controls were the sections reacted with antiserum to the PSbMV cylindrical inclusion protein.

3.3 RESULTS

3.3.1 Whole Virus

Plate 3.1 shows PSbMV in infected pea material. The particles are approximately 11 nm wide and 780 nm long. The flexuous nature of the potyvirus particle is evident.

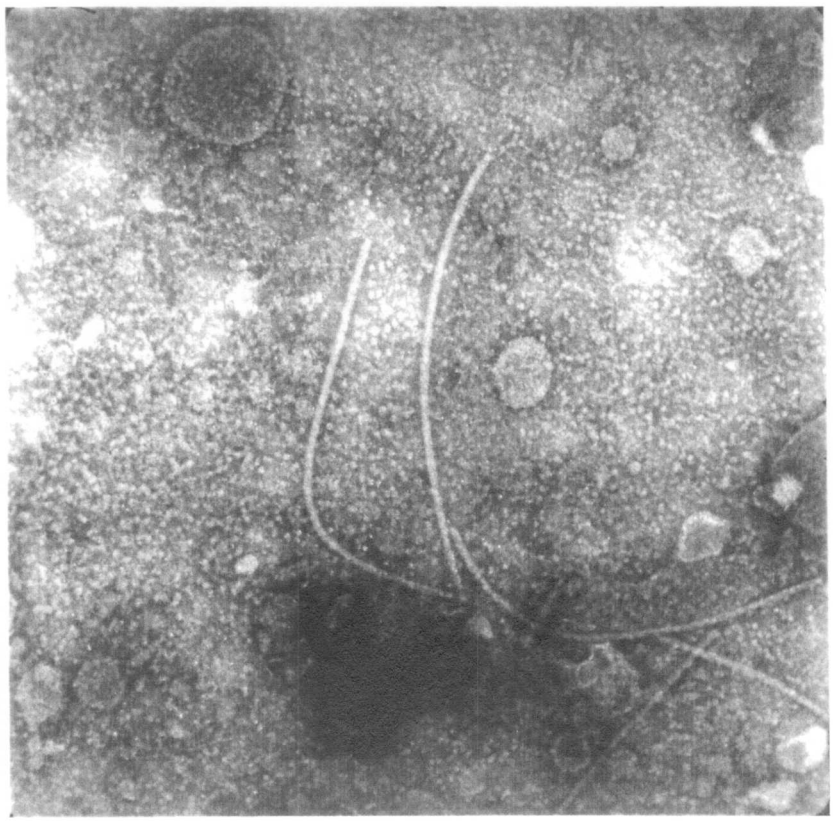


Plate 3.1. Virus particles in infected leaf tissue stained with PTA.

The virus particles are approximately 780 nm long and 11 nm wide.

Plates 3.2-3.5. The morphologies observed for the CIs of PSbMV in sections of pea leaves and roots.

Plate 3.2. Root section showing bundles (b) and pinwheels (p).

Plate 3.3. Leaf section showing scrolls (s).

Plate 3.4. Leaf secton showing pinwheels (p) and scrolls (s).

Plate 3.5. Root section showing pinwheels (p).

Plate 3.2

Plate 3.3

Plate 3.4

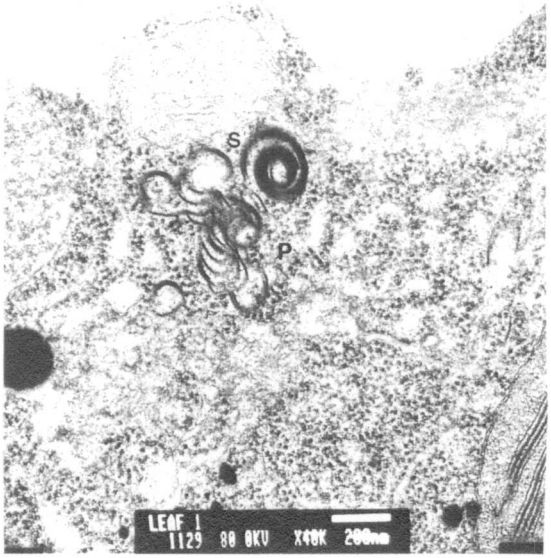


Plate 3.5

Plates 3.6-3.9. These plates show the close association between the plasmodesmata and the CIs.

Plate 3.6. Pinwheels (p) associated with the plasmodesmata (pl) in a pea root. The plasmodesmata are shown in cross-section.

Plate 3.7. A section through a pea leaf showing the association of CIs with the cell wall (cw). The plasmodesmata are in longitudinal section.

Plate 3.8. The CIs in two adjacent cells are observed to be associated with the plasmodesmata (pl). The root section shows the plasmodesmata in longitudinal section.

Plate 3.9. CIs orientated perpendicular to the plasmodesmata (pl). The leaf section shows the plasmodesmata in longitudinal section. The cell wall (cw) of the two adjacent cells are indicated.

Plate 3.6

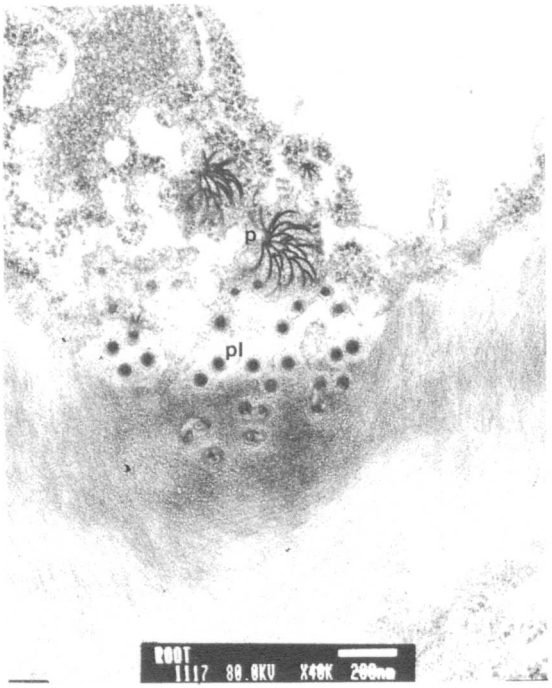


Plate 3.7

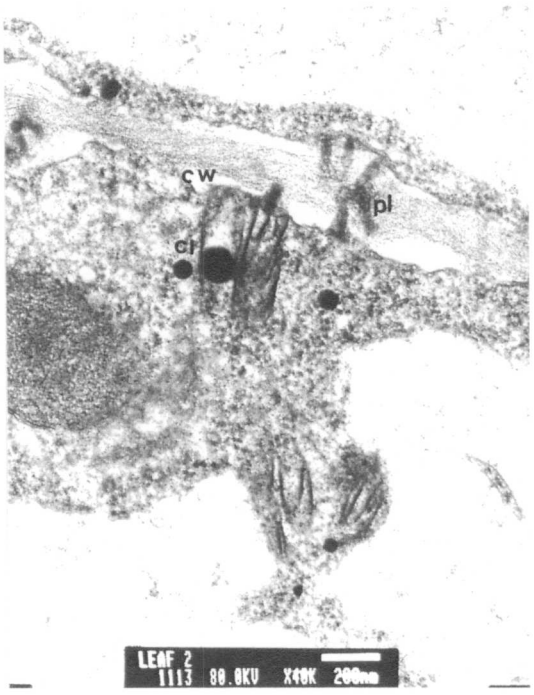


Plate 3.8

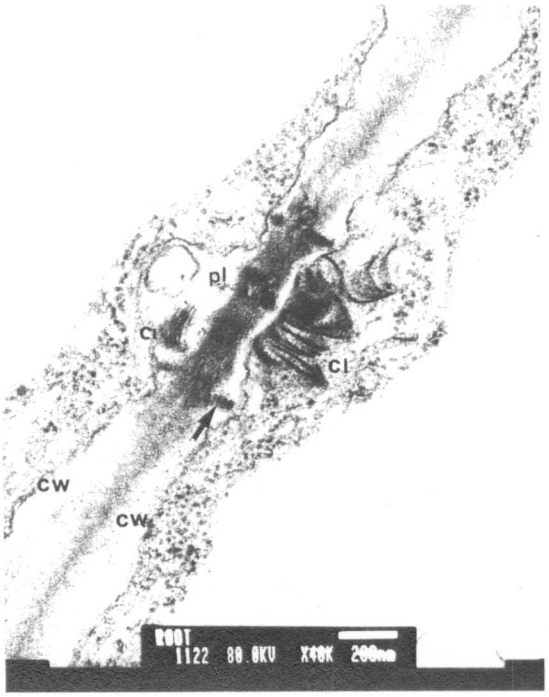


Plate 3.9

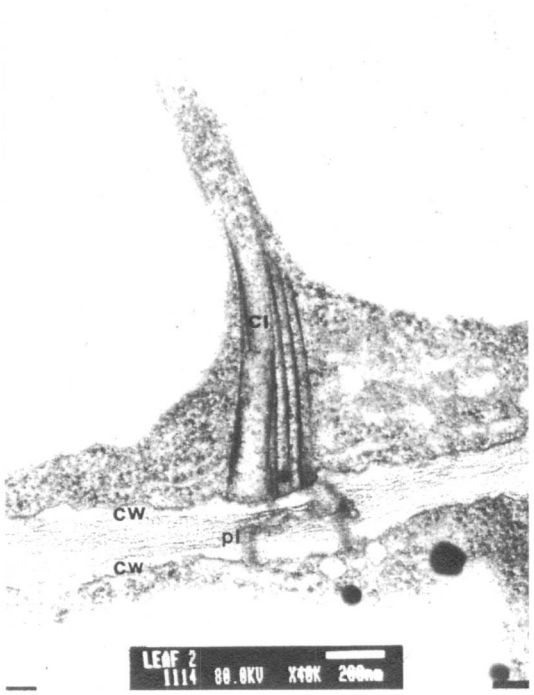


Plate 3.10. A leaf section showing short curved laminated aggregates (la).

Plate 3.11. Root section showing the association between the bundles (b) and the rough endoplasmic reticulum (rER).

Plate 3.10

Plate 3.11

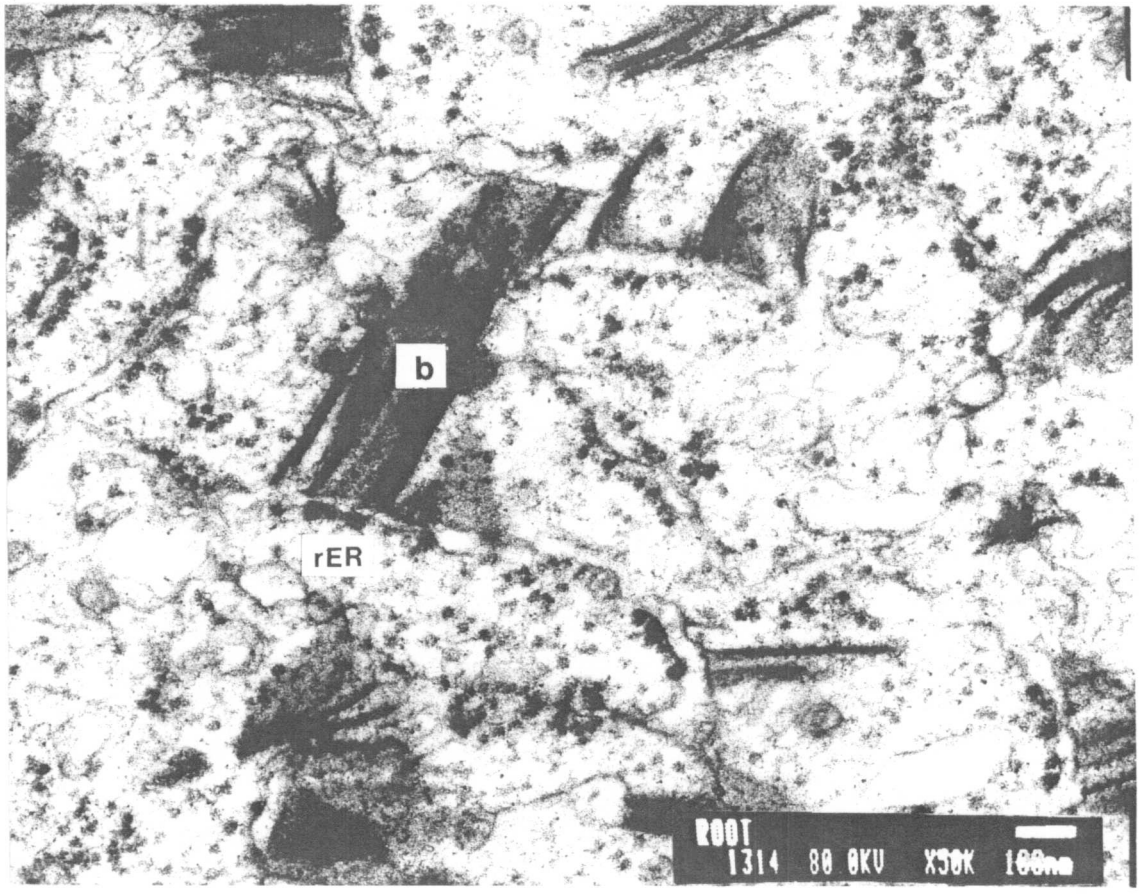


Plate 3.12. Infected pea protoplasts reacted with antiserum to PSbMV CP (1° antiserum) and donkey anti-rabbit conjugated to fluorescein isothiocyanate (2° antiserum). The arrow indicates the material that fluoresced green when viewed using a fluorescent microscope. Magnification x 400.

Plate 3.13. Control of uninfected protoplasts treated as in plate 3.12. Magnification x 400.

Plate 3.14. This shows infected pea protoplasts reacted with antiserum to PSbMV CI protein (1° antiserum). The arrow indicates the material that fluoresced green. Magnification x 400.

Plate 3.15. Control of uninfected protoplasts treated as in plate 3.14. Magnification x 400.

Plate 3.12

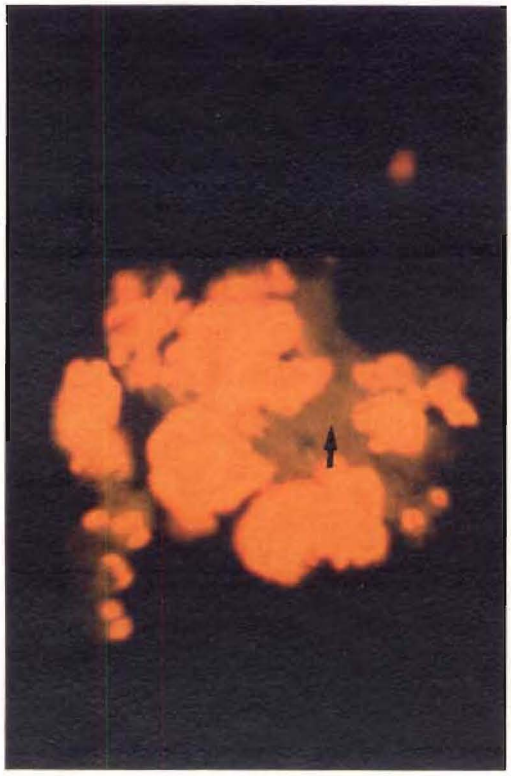


Plate 3.13

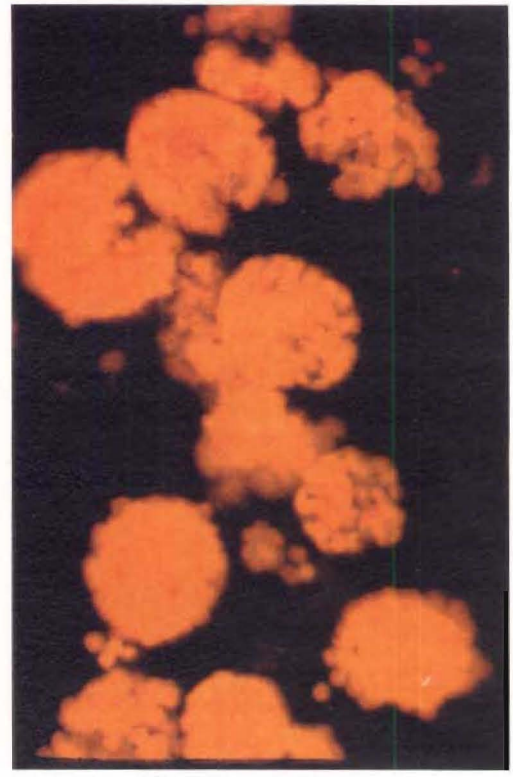


Plate 3.14

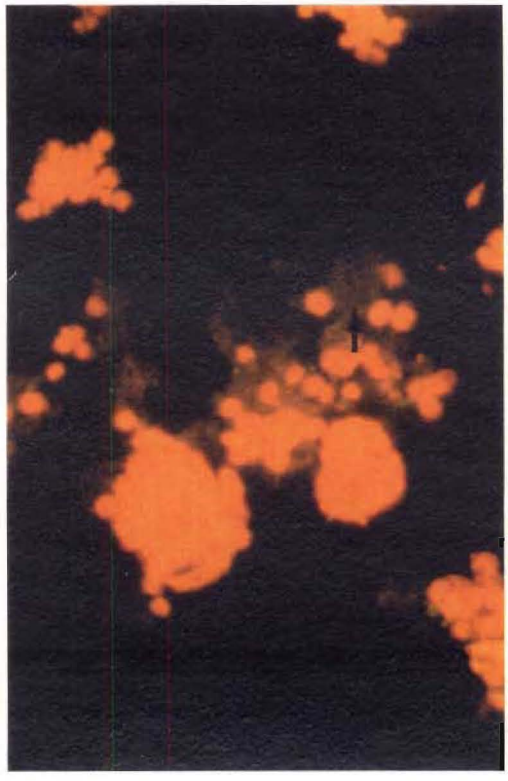
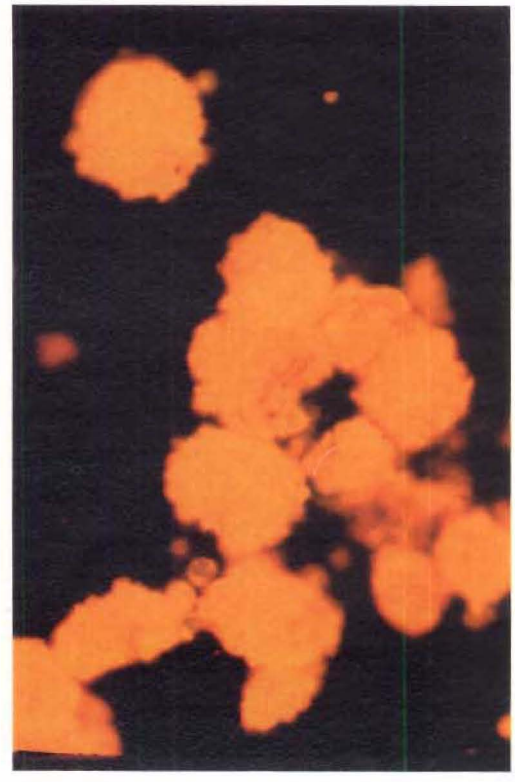


Plate 3.15



3.3.2.Inclusions

Edwardson (1974) and Edwardson *et al.* (1984) proposed that the different morphologies observed for the CIs could be used to classify potyviruses. PSbMV had been assigned to subdivision-III (Edwardson 1974). However, viruses in subdivision III induce long aggregated laminates (as well as pinwheels and scrolls). Long aggregated laminates were not observed in tissue infected with PSbMV. Instead short curved laminated aggregates were observed. The short curved laminated aggregates are induced by viruses in subdivision-IV, not subdivision III.

Three morphologies of the CIs of PSbMV were observed. Plate 3.2 shows pinwheels (bundles when seen in longitudinal section) and scrolls (tubes in longitudinal section) in a section of pea root. Plates 3.3 and 3.4 show the presence of scrolls and/or pinwheels in leaf sections. Plate 3.5 shows pinwheels in a root section. The short curved laminated aggregates were seen only infrequently, and then only in leaf sections. Plate 3.10 shows laminated aggregates in a leaf section. A general impression of the relative abundance of pinwheels and scrolls in leaf tissue compared with root tissue suggests that the two contain approximately equal numbers of inclusions. A large number of cells in both types of tissue did not contain any CIs, but the cells that did contained an abundance of them.

Several workers, for example Langenberg (1986), have noted the association between the CIs and plasmodesmata. An association between the CIs of PSbMV and host plasmodesmata can be seen in Plates 3.6-3.9. Plate 3.6 shows the plasmodesmata in cross-section. The pinwheel, indicated with a 'p', is seen to have a close association with a plasmodesma. The other plates (3.7-3.9) show the plasmodesmata in longitudinal section. Plate 3.9 shows clearly how the CIs "line-up" with the plasmodesmata. In Plate 3.8 CIs appear to be localised to regions near the plasmodesmata. The CIs of both cells shown in this plate are associated with the plasmodesmata. No CIs are evident further along the cell wall in either direction.

The pinwheel (and bundle) form of the CI was observed to be consistently associated with rough endoplasmic reticulum (rER). Plates 3.2 and 3.11 show the truncated bundles closely associated with rER.

No amorphous or nuclear inclusions were observed in the sections of either leaves or roots.

3.3.3 Immunofluorescence

Filters that absorb the fluorescence of chlorophylls a and b were not available. This presented some difficulty as the fluorescence of the fluoresceln was masked by the bright fluorescence of the chlorophylls. The interpretation of positive or negative fluorescence relied upon lysing some of the protoplasts. The cell membrane functioned to retain the chloroplasts, while the cytoplasm was able to extrude through breaks in the cell membrane. While this technique is involved, the results of the "blind" experiments verify its reliability.

Approximately 25% of the protoplasts isolated from infected peas showed fluorescence when labelled with PSbMV CP or PSbMV CI antiserum as the 1° antisera. Plate 3.12 shows infected protoplasts reacted with PSbMV CP as the 1° antiserum. The material, indicated by an arrow, actually appeared green when viewed with the microscope. The fluorescence was actually quite bright and no difficulty was experienced in deciding whether the protoplasts were fluorescing or not. The difference between the infected protoplasts (Plate 3.12) and the uninfected protoplasts (Plate 3.13) can be seen. A similar result was observed when PSbMV CI antiserum was used. Plate 3.14 shows infected protoplasts incubated with PSbMV CI as the 1° antiserum, and Plate 3.15 shows uninfected protoplasts also treated with PSbMV CI as the 1° antiserum.

3.3.4 Attempted Localisation of the Nuclear Inclusions

Three different techniques (immunofluorescence, Western blots and immunogold labelling) were used in an attempt to localise the nuclear inclusion protein(s). None of the techniques gave satisfactory answers. Observing the immunofluorescence of specific proteins was hampered by the unavailability of filters to absorb the fluorescence of the chlorophylls a and b. While lysed cells gave satisfactory results for PSbMV CP and PSbMV CI, these two proteins occurred in the cytoplasm and are found as large structures - the CP as part of the virion and the CI protein as inclusions. The NI proteins were not observed to form aggregations in PSbMV infected cells. This may mean that fluorescence from the solubilised protein is not bright enough to see. In addition, if the NI proteins are localised in the nuclei of infected cells, the nuclei themselves may not have been extruded from the lysed cells but may have been held within the bounds of the plasmamembrane together with the chloroplasts.

The use of Western blots to localise the NIb protein of PSbMV to either the nuclear or cytoplasmic fractions of infected cells was also unsuccessful. Intact pea nuclei were obtained (shown by microscopic examination, data not presented), but all preparations contained debris. The presence of this contaminating debris, coupled with the sensitivity of the Western blot technique, means that the data were unreliable. In addition to the debris, contaminating material may be derived from lysed nuclei or from leakage of nuclear material through the nuclear pores.

The samples from both the nuclear and cytoplasmic fractions of infected pea leaves (Fig.3.1, lanes 2 and 4) showed several bands. These had molecular weights of 63 kDa, 48 kDa, and 38 kDa. The middle band, with a molecular weight of 48 kDa, may correspond to the NIb protein. The NIb protein of PSbMV was determined to have a molecular weight of approximately 48 kDa (see Chapter 4). Lanes 1 and 3 represent material from uninfected pea nuclear fraction and uninfected pea cytoplasmic fraction, respectively. These two lanes showed the presence of a single band with a molecular weight of approximately 38 kDa. This band is apparent in all four samples. The two larger bands seen in lanes 2 and 4 appear to be associated with infected pea

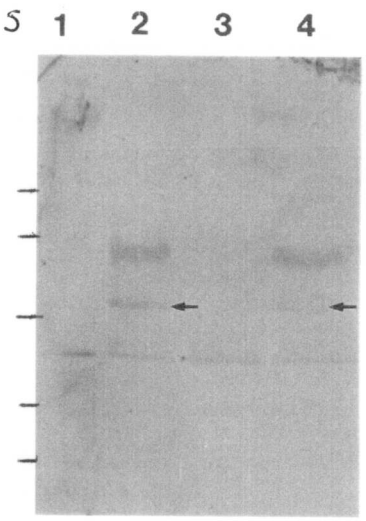


Fig.3.1. Western blot analysis of nuclear and cytoplasmic fractions from leaves and reacted with 1° antiserum to TEV NIb protein. **Lane 1.** Nuclear fraction from uninfected pea. **Lane 2.** Nuclear fraction from infected pea. **Lane 3.** Cytoplasmic fraction from uninfected pea. **Lane 4.** Cytoplasmic fraction from infected pea. Lane 5. Markers (Biorad). Rabbit muscle phosphorylase b (97,000), BSA (66,000), hen egg white ovalbumin (42,699), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and hen egg white lysozyme (14,400).

material. The identity of the 63 kDa and the 38 kDa proteins remain obscure. Because of the presence of multiple bands in lanes 2 and 4 (from infected material), no conclusions can be drawn regarding the locality of the NIb protein.

Immunogold labelling, the third technique which attempted to localise the nuclear inclusions also gave inconclusive results. The gold was not localised to any particular part of the cell (data not presented). The control experiment using PSbMV CI antiserum did not localise the gold to the CIs observed in the cytoplasm of infected cells (data not presented). The problems may have been due to the embedding medium. Spurr's resin requires heating to 70°C overnight to polymerise. The heating step possibly destroys the secondary structure of the protein so that the antiserum does not recognise it.

3.4 DISCUSSION

The approximate dimensions of 780 nm long by 11 nm wide observed for PSbMV in this study are in agreement with those of Hampton and Mink (1975), who reported a modal length of 770 nm and a diameter of 11 nm for PSbMV. The number of particles appearing in each microscope field is low and indicates the scarcity of particles in infected sap. The low concentration of virus in infected sap was discussed in Chapter 2 as a characteristic of potyvirus infections (Stevenson and Hagedorn 1973).

The various morphologies observed for the cylindrical inclusion bodies place PSbMV in subdivision-IV according to the classification of Edwardson *et al.* (1984). In 1984, 17 viruses were assigned to this group, five infecting primarily monocotyledons and 12 primarily dicotyledons (Edwardson *et al.* 1984). Watermelon mosaic virus (WMMV) and pepper mottle virus (PeMV) are included in the subdivision.

Serological, biological and biochemical properties have all been used in the classification of potyviruses (Abu-Samah and Randles 1981; Edwardson 1974 and Moghal and Francki 1976). Clarification of the taxonomy of the group is considered by many to be overdue (Harrison 1985; Hollings and Brunt 1981). It would appear that grouping potyviruses into subdivisions, as proposed by Edwardson (1974) and Edwardson *et al.* (1984), could complement the protein sequence analysis done predominantly by Shukla and colleagues (for example Shukla and Ward 1989b; Frenkel *et al.* 1989 and Shukla *et al.* 1987). Shukla and colleagues have used sequence homology data from N-terminal amino acids of the coat protein of various potyviruses to define which viruses are strains and which are distinct members of the potyvirus group. Several key viruses indicate the complementary nature of cylindrical inclusion morphologies and sequence data for the classification and taxonomy of potyviruses. One example is that of PeMV and potato virus Y (PVY). Edwardson *et al.* in 1984 placed both viruses into subdivision-IV. At this time they were considered to be distinct viruses. Later, Shukla *et al.* (1988b) using amino acid sequence data designated PeMV as a strain of PVY. In a second example, sugar cane mosaic virus (SCMV)

and Johnsongrass mosaic virus (JGMV) were originally recognised as strains of the same virus [JGMV was originally termed SCMV Johnsongrass strain (SCMV-JG strain)]. However, Edwardson (1974) put these two viruses into different subdivisions. JGMV (then called SCMV-JG strain) was put into subdivision-I while SCMV was placed in subdivision-III [SCMV has subsequently been placed into subdivision-IV (Edwardson *et al.* 1984)]. In 1987 Shukla *et al.*, compared the N-termini amino acid sequences of the coat proteins of SCMV and SCMV-JG strain. As a result they proposed JGMV be recognised as a distinct member of the potyvirus group and coined the name JGMV. These two examples serve to illustrate that subdivision of the group using cylindrical inclusion morphology is not in conflict with the use of amino acid sequence data to distinguish distinct viruses from strains. Indeed, it would appear that subdivisions proposed by Edwarson (1974) have given some useful clues to the relatedness of some viruses that were later clarified by amino acid sequence data.

Serology has also been suggested as being useful in determining the relatedness of potyviruses (Shukla and Ward 1989a). While the technique shows promise, unexplained paired serological relationships between distinct viruses, for example TEV and SCMV (Shukla and Ward 1989b), must be considered when using serology to classify potyviruses. Francki (1983) noted that potyviruses that are antigenically related do not share most of their other properties. This is in contrast with other virus families. As a result, serology, while being a quick, easy technique to use, should be treated with caution when applied it to the taxonomy of potyviruses.

Several functions have been suggested for the cylindrical inclusions of potyviruses. Langenberg (1986) proposed that the protein may be involved in the cell-to-cell spread of the virus. His proposal is an attempt to rationalise observations relating to the position of the cylindrical inclusions adjacent to the plasmodesmata and the association of the virus coat protein with these inclusions. Because of the long flexuous nature of the virions, Langenberg suggested that their alignment for transport through the plasmodesmata, observed for example by McMullen and Gardner (1980), must be an active process which is possibly facilitated by the cylindrical inclusion bodies. This is also supported by the earlier observations of Lawson *et al.* (1971), who showed that the cylindrical inclusions of PVY are associated with the plasmodesmata, and by my own observations.

The cylindrical inclusion protein may also play a role in replication analogous to that of the P2C protein of picornaviruses. Domier *et al.* (1987) compared the predicted amino acid sequence of TEV and TVMV to proteins from poliovirus and encephalomyocarditis virus (both picornaviruses) and the B RNA of cowpea mosaic virus (CPMV). The CI protein showed significant sequence homology to the P2C proteins of picornaviruses and to the 58 kDa protein encoded by the B RNA of CPMV. More specifically, a highly conserved sequence of GXXGXGKS was found in all the above viruses. Evidence for the role of the P2C protein of picornavirses has come from electron microscopic immunocytochemistry and autoradiography. Bienz *et al.* (1987)

localised the P2C protein of poliovirus to vesicles and postulated that the likely function of the P2C protein is to attach all or part of the replication complex to the vesicular membranes. Domier *et al.* (1987) suggested that by analogy the cylindrical inclusion protein of potyviruses is the membrane-binding component of the replicase complex. In addition to finding the highly conserved sequence in the viruses, Domier *et al.* (1987) also noted a similar region is found in the *E. coli* EF-Tu protein. The highly conserved sequence of GXXXXGK in EF-Tu is suggested to form part of the binding site for the phoshoryl portion of nucleoside triphosphates (la Cour *et al.* 1985). The proposed sequence similarity to EF-Tu is consistent with any function requiring large amounts of energy (for example transport). The cylindrical inclusion protein may eventually be shown to be involved in both cell-to-cell spread and in replication, as it would appear that the 70 kDa cylindrical inclusion protein is large enough to have more than one active domain.

The cylindrical inclusions of PSbMV are observed to be consistently associated with the rER. Several other workers have observed a similar association, for example Krass and Ford (1968), Lawson *et al.* (1971), McMullen and Gardner (1980). The association can also be seen in Fig. 4 in Edwardson and Christie (1983), although the authors did not comment on it. The reason for the association has not been elucidated.

The absence of nuclear bodies, despite the fact that the virus is believed to code for and induce the appropriate proteins in infected plants, remains an enigma. No nuclear inclusions were present in either the leaf or root cells of peas infected with PSbMV, yet Western blots of infected material have shown that one protein that comprises the nuclear inclusion (NIb) is present (Chapter 4). In addition, both the NIa and NIb proteins were immunoprecipitated from rabbit reticulocyte lysate programmed with PSbMV-RNA. Attempts to localise one of the nuclear inclusion proteins (NIb) using Western blots, immunogold labelling and immunofluorescence, were unsuccessful. It may be that aggregations in the nuclei induced by viruses like TEV are localised to a specific site (in this case, the nucleus) because their large size does not permit movement through the nuclear pores. Just why these proteins aggregate specifically in the nuclei of these cells remains unknown. The reason why some members induce inclusions in their hosts, while others do not, is also unknown. Some of these questions may be answered when further studies into virus-host interactions are conducted.

In conclusion, PSbMV is classified in subdivision-IV, based on the induction of scrolls, pinwheels and short curved laminated aggregates. In addition, the cylindrical inclusions of PSbMV appear to be associated with both the plasmodesmata and the rough endoplasmic reticulum.

CHAPTER 4

ESTABLISHING THE MOLECULAR WEIGHTS OF 5 VIRUS-ENCODED PROTEINS USING WESTERN BLOTS

4.1 INTRODUCTION

The present chapter describes how Western blotting was used to establish the previously unknown molecular weights of four PSbMV-encoded proteins and to confirm the molecular weight of the coat protein, previously established by SDS-PAGE.

Towbin *et al.* (1979) devised a method (Western blotting) to electrophoretically transfer proteins from polyacrylamide gels to nitrocellulose sheets. From the outset, a variety of methods for detecting the position of the immobilised proteins were used (Towbin *et al.* 1979). The most widely used detection system involves the use of a secondary antibody conjugated to an enzyme, usually alkaline phosphatase or horse-radish peroxidase. The choice between these two enzymes depends on a number of factors. First, the sensitivity of antibodies conjugated to alkaline phosphatase allows them to detect 10-50 picograms of protein (Biorad blotting manual), whereas horse-radish peroxidase is 10-20 fold less sensitive, detecting only 250-500 picograms of protein. In my study, 2.5 ng of protein was easily detected. Second, the stability of the substrate reagents favours alkaline phosphatase. The substrates for the horse-radish peroxidase assay are unstable and must be prepared freshly each time. Moreover, they are carcinogenic (Biorad blotting manual) and fade quickly in the presence of light. This precludes storing the blots for any length of time. In contrast, the substrates for alkaline phosphatase are stable, may be stored as stock solutions, are safe to handle and give a clear colour reaction that does not fade over time.

The technique has a wide range of applications in animal biology. For example, Towbin *et al.* (1979) used the method to detect ribosomal proteins and suggested other uses, including one-dimensional finger-print studies. The technique has been used infrequently by plant virologists, although van Pelt-Heerschap *et al.* (1987) used Westerns to detect viral proteins in protoplasts and Zabel *et al.* (1982) employed them to detect the presence of the VPg in the polyprotein of cowpea mosaic virus (CPMV).

A number of functions have been proposed for 5 potyvirus- encoded proteins. The only structural protein is the coat protein which envelops the genomic material with approximately 2000 monomeric units (Hollings and Brunt 1981). Each potyvirus contains only a single type of capsid protein monomer, ranging in size from 30-45 kDa (Allison *et al.* 1985). The assembled potyvirus capsid proteins appear to have the amino- and carboxyl-terminal segments of the coat

protein on the surface of the virion (Shukla *et al.* 1988a). This organisation is similar to that of potexviruses (Sawyer *et al.* 1987) and tobamoviruses (Holmes 1980).

PSbMV, like other members of the potyvirus group, codes for a number of non-structural proteins. One such protein, the cylindrical inclusion protein has been discussed in sections 3.1 and 3.4.

A second non-structural protein encoded by potyviruses is the amorphous inclusion protein. The amorphous inclusion, so named because it occurs as large amorphous aggregations in the cytoplasm of some plants infected with some potyviruses, has been identified as a distinct viral protein (Pirone and Thornbury 1983; de Mejia *et al.* 1985b). There is some debate as to whether the amorphous inclusion protein is synonomous with the helper component (discussed in Dougherty and Carrington 1988). Govier *et al.* (1977) and Govier and Kassanis (1974) proposed that the helper component is associated with the transmission of the virus by its insect vector. This observation has been supported more recently by the work of Berger and Pirone (1986) with TEV and PVY. Their study found that ^{125}I label, and by analogy TEV and PVY virions, were specifically associated with parts of the aphid gut only when helper component was present in the feeding solution. But, helper component appeared to have no effect upon the uptake of the virus from infected sap. Pirone and Thornbury (1983) came to similar conclusions using TEV. Some questions still remain, however. This is mainly due to observations by T. Pirone (pers.comm. in Dougherty and Carrington 1988) that purified amorphous inclusion bodies do not have helper component activity. Two explanations offer themselves - either the inclusion bound form is inactive or some further processing is required to produce an active form (Dougherty and Carrington 1988).

In a limited number of potyvirus infections, two distinct proteins aggregate in equimolar amounts (Dougherty and Hiebert 1980b) to form stable nuclear inclusion bodies (Knuhtsen *et al.* 1974). The aggregates vary in size and shape and they are specific to particular virus isolates (Christie and Edwardson 1977). Hellmann *et al.* (1988) reported modified *in vitro* "hybrid arrest" translations of TVMV RNA and translations of *in vitro* transcripts of cDNA encoding the TVMV NIa protein. This work provided evidence that the smaller 49 kDa (NIa) protein is a virus-encoded protease which is responsible for cleaving the viral polypeptide at several sites. The study of Carrington and Dougherty (1987a, 1987b) on TEV supports this proposed function for the NIa protein. Further evidence that the NIa protein is a putative protease comes from the study of Chang *et al.* (1988). In their study, antiserum to the NIa protein of bean yellow mosaic virus (BYMV) was included in *in vitro* translations of BYMV RNA in rabbit reticulocyte lysate. Inclusion of the antiserum in the translation mix resulted in the accumulation of high molecular weight precursors, indicating a role of a putative protease for the NIa protein. The function of the larger 58 kDa NIb protein is less clear. On the basis of sequence similarity studies between

picornaviruses, CPMV (comovirus) and two potyviruses (TEV and TVMV), Domier *et al.* (1987) proposed that the 58 kDa protein is an RNA dependent RNA polymerase (a viral replicase).

4.2 MATERIALS AND METHODS

Infected peas were propagated and inoculated as described in Chapter 2.2.

The sources of the 7 antisera to different viral-encoded proteins are outlined in Table 3.1.

4.2.1 Preparation of Crude Sap for Immunological Analysis

0.1 g of plant material was homogenised in 800 μl PBS (4 mM KH_2PO_4, 16 mM Na_2HPO_4 and 115 mM NaCl) pH 7.4 in a glass tissue grinder. The sample was centrifuged at 5,000 g for 5 minutes and the supernatant was collected. For immunodot blots, 5 μl of supernatant was then diluted to 200 μl with PBS, pH 7.4.

4.2.2 Comparison of 3 brands of Nitrocellulose Membrane (NCM)

Immunodot blots were employed to test the relative ability of 3 different brands of NCM to bind proteins: Schleicher and Schull, Gelman, and Biorad. All had pore sizes of 0.45 μm.

Using the Biorad slot blot apparatus, duplicate 200 μl samples of maize dwarf mosaic virus (MDMV) coat protein antiserum diluted 1:500 in PBS, pH 7.4, were applied to the 3 different brands of NCM according to the manufacturers' instructions. Positive control slots contained goat anti-rabbit antiserum conjugated to alkaline phosphatase (2° antiserum) at a dilution of 1:500. The membranes were removed from the apparatus and blocked with 20 ml PBS/Tween containing 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.3,and 0.05% Tween 20 (Batteiger *et al.* 1982) at 25°C for one hour with constant agitation. The membranes were then drained and sealed in a plastic bag containing 2 ml of 2° antiserum at a dilution of 1:500. This was incubated at 25°C for two hours with vigorous shaking, facilitated by removing the holding racks and some water from a shaking water bath and taping the plastic bags onto the shaking platform. The membranes were then washed 3 times for 5 minutes each with PBS/Tween and incubated with a modification of the substrates originally described by McGadey (1970) and more recently by Blake *et al.*(1984) and Leary *et al.* (1983). The substrate was composed of two main components, a 5 mg ml^{-1} solution of 5 bromo-4-chloroindoyl phosphate (BCIP) in dimethylformamide and a 0.1% solution of nitro blue tetrazolium (NBT) in 0.15 M veronal acetate buffer, pH 9.6. The substrate solution contained 20 μl 2 M $MgCl_2$, 1 ml NBT, 0.1 ml BCIP and 9 ml 0.15 M veronal acetate buffer pH 9.6. As soon as the colour developed (normally within 15 minutes), the membranes were removed, rinsed with dH_2O and air-dried.

4.2.3 Concentration of Primary and Secondary Antisera

Immunodot blots were employed to test the optimal dilutions for the 1° and 2° antisera. A grid system was set up to test a number of parameters (Fig 4.2).

Plant extracts were prepared as outlined in section 4.2.1. Uninfected plant extracts were prepared in the same manner and were used as controls.

Duplicate samples of 200 μl of plant extract were applied to the NCM as described in section 4.2.2. The membrane was blocked with PBS/Tween as described in section 4.2.2. Each slot was treated with the appropriate 1° and 2° antisera as indicated in Fig.4.2 at a volume corresponding to 0.1 ml per cm^2 of NCM (Ey and Ashman 1986). The application of the 1° antisera: PSbMV CP, PSbMV CI or TEV NIb, and the 2° antiserum follows the procedure outlined in section 4.2.2. For NCM strips treated with the 1° antisera to TEV NIa, WMMV AI or PeMV AI, extra stringency was employed as preliminary results indicated a high level of non-specific binding. The extra stringency for the NCM incubated with these 1° antisrea comprised agitating the membrane with the 1° antiserum at 37°C instead of at 25°C, which was used for the other antisera. Each of the 6 different 1° antisera and the 2° antiserum was tested. Control slots containing 200 μl of 1:500 dilution of 2° antiserum applied directly to the NCM were included.

4.2.4 Transfer of Proteins onto NCM (Biorad manual)

Soluble extracts of plant material for Western blot analysis were prepared using 0.1 g of leaf material as outlined in section 4.2.1.

The 20 μl samples were prepared for electrophoresis by making the appropriate dilution of 5 x sample buffer (SB) containing 0.3 M Tris-HCl pH 6.8, 10% SDS, 62.5% (w/v) glycerol, 3.5 M β mercaptoethanol and 0.004% BPB. The samples were heated at 100°C for 2 minutes, followed by cooling on ice. 5 μl of sample was loaded onto the gel.

An 8 ml SDS polyacrylamide gel (Laemmli 1970) containing 10% acrylamide, 0.1% SDS, 0.1% ammonium persulphate (APS) and 4 μl TEMED in 0.37 M Tris-HCl pH 8.8 was prepared. The 5 ml stacking gel contained 3% acrylamide, 0.1% SDS, 0.04% APS, 7.5 μl TEMED in 0.125 M Tris-HCl pH 6.8. The gel was 1.5 mm thick.

Electrophoresis buffer contained 30 mM Tris, 240 mM glycine and 0.125% SDS, pH 8.3. The gels were electrophoresed at 6 mA constant current through the stacking gel and 3 mA through the separating gel.

Following electrophoresis, the gel was soaked in transfer buffer (20 mM Tris, 192 mM glycine and 20% (v/v) methanol) for 15 minutes to equilibrate. A "sandwich" was constructed as follows: First, a fibre pad was soaked in transfer buffer and placed on the opened transfer cassette. On top of this, was placed a piece of 3MM Whatman filter paper cut to the same size as the gel and also soaked in transfer buffer. The equilibrated gel was positioned on the filter paper taking care to exclude air bubbles. NCM (Gelman) was carefully wetted with the transfer buffer

and placed on top of the gel, again taking care to exclude air bubbles. This was followed by another piece of wetted filter paper and another fibre pad.The cassette was carefully closed and lowered into the transfer tank with the nitrocellulose side facing the anode. The cassette was completely immersed in transfer buffer. Proteins were transferred at 100 volts and 250 mA for one hour. As heating was a problem, the contents of the tank were cooled by placing a cell containing ice in the transfer tank and stirred constantly during transfer to distribute the heat.

The efficiency of transfer was investigated by staining the gel with Coomassie Blue after transfer. The gel was gently agitated in 100 ml Coomassie Blue R250 (50% methanol, 10% glacial acetic acid and 0.1% (w/v) Coomassie Blue R250) for 30 minutes. This was followed by destaining in 500 ml 5% methanol and 10% glacial acetic acid for 4 hours with shaking, with a destain change after 30 minutes.

4.2.5 Blocking and Washing Solutions

Three different blocking solutions were tested for their ability to minimise non-specific binding. PSbMV CP antiserum was used as a representative 1° antiserum. Following transfer, the NCM was blocked with one of three blocking solutions. The first was Tris buffered saline (TBS) with 3% non-fat milk powder (30 mM Tris HCl, pH 7.4, 150 mM NaCl, 3% non-fat milk powder and 0.1% Tween 20). The second was TBS plus 1% bovine serum albumin (BSA), and the third was phosphate buffered saline with Tween 20 (PBS/Tween) containing 4mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, 0.05% Tween 20 (Batteiger *et al.* 1982) and 3% non-fat milk powder.

Blocking involved agitating each of the NCMs in 10 ml of one of the blocking solutions for two hours at 25°C. The membranes were washed 3 times for 5 minutes each with the appropriate washing solution consisting of blocking solution without the milk powder or BSA. The membranes were then drained and sealed in a plastic bag containing 0.1 ml per cm^2 of NCM (Ey and Ashman 1986) of PSbMV coat protein antiserum 1:4000 in PBS/Tween plus 3% BSA. The rest of the procedure was the same as for the immunodot blots (see section 4.2.2).

4.2.6 Staining the Markers

Low molecular weight protein markers (Biorad) were prepared according to the manufacturer's instructions. One μl of marker solution was diluted to 20 μl in a 1 x SB, heated at 100°C for two minutes and cooled on ice. 10 μl was loaded onto the gel.

The markers were transferred onto the NCM along with the sample lanes. The membrane was blocked with PBS/Tween for one hour at 25°C with shaking. Three different stains were compared for their ability to stain the transferred markers. For the first, the marker lane NCM was gently agitated in 10 ml Coomassie G250 (0.2 g Coomassie Brillant Blue G250, 200 ml phosphoric acid and 800 ml H_2O, Bradford 1976) for 30 minutes. If the markers were not clear,

the membrane was destained for two to 3 hours in 5% glacial acetic acid with 3 changes of destain during this period. The membrane was finally rinsed in dH_2O and air-dried.

The second method used Coomassie Blue R250 as the stain. The method for staining the NCM was essentially the same as outlined above for staining gels.

The third method used amido black 10-B (Schaffner and Weissmann 1973). The marker lane NCM was gently agitated in 10 ml 0.1% (w/v) amido black in 45% (v/v) methanol and 10% (v/v) glacial acetic acid. The membrane was drained and destained for no more than 5 minutes with 90% (v/v) methanol, 2% (v/v) glacial acetic acid in dH_2O, then washed with dH_2O.

4.2.7 Molecular Weights of the Virus Encoded Proteins

Western blots were carried out as outlined in section 4.2.4 to establish the molecular weights of the 5 virus-encoded proteins for which antisera were available. 5 μl of infected plant material in 1 x SB (section 4.2.4) was loaded onto the gel, electrophoresed and transferred onto NCM. The membrane was blocked with PBS/Tween containing 3% milk powder and washed 3 times for 5 minutes each in PBS/Tween. The individual strips of NCM, each corresponding to a single lane on the gel, were then agitated overnight at 25°C in 1:4000 dilution of the appropriate 1° antiserum. On the following day, the membrane was drained, washed as before and agitated for two hours at 25°C in 1:500 dilution of 2° antiserum. This was followed by another wash, then the membrane was drained and reacted with 10 ml substrate as described in section 4.2.2. After colour development, the membranes were rinsed in dH_2O and air-dried. Controls of uninfected plant material were tested in the same manner as described above.

4.3 RESULTS

4.3.1 Comparison of Nitrocellulose Membranes

Antiserum to the coat protein of MDMV was used as a representative protein to determine which of three brands of NCM bound proteins most efficiently under the conditions used in these experiments. Of the brands tested, namely Schleicher and Schull, Biorad, and Gelman (Bio Trace), the membrane supplied by Gelman showed a significantly higher binding capacity for proteins than the other two brands (Fig.4.1). The higher binding capacity was judged by the intensity of the indigo dye produced in response to the reaction of alkaline phosphatase on the substrates BCIP and NBT. All subsequent electroblotting was conducted using "Bio Trace" by Gelman.

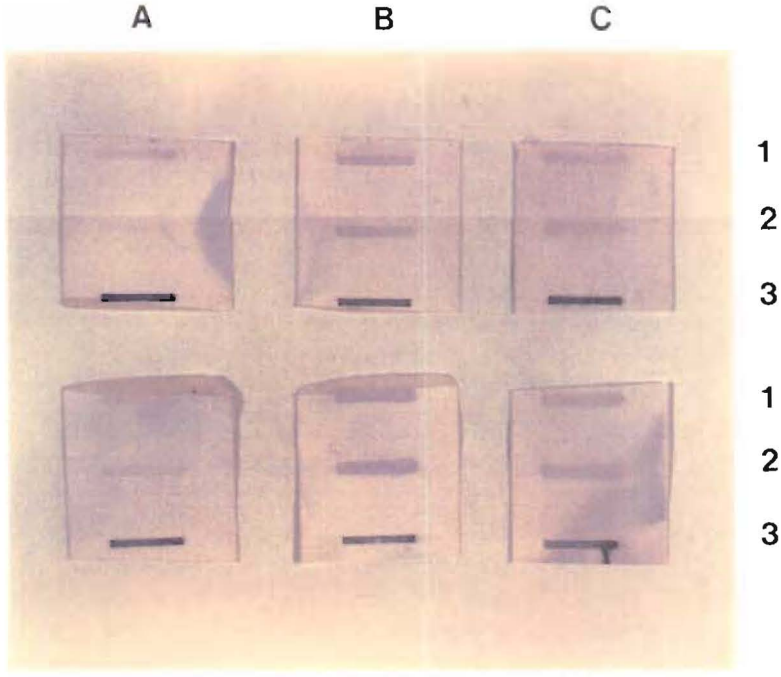


Fig.4.1. A comparison of 3 brands of nitrocellulose. **A**. Schleicher and Schull. **B**. Gelman. **C**. Biorad. Rows 1 and 2. 200 ul of antiserum to the CP of MDMV at 1:500 dilution. Row 3. 2º antiserum at 1:500 dilution. The results are shown in duplicate.

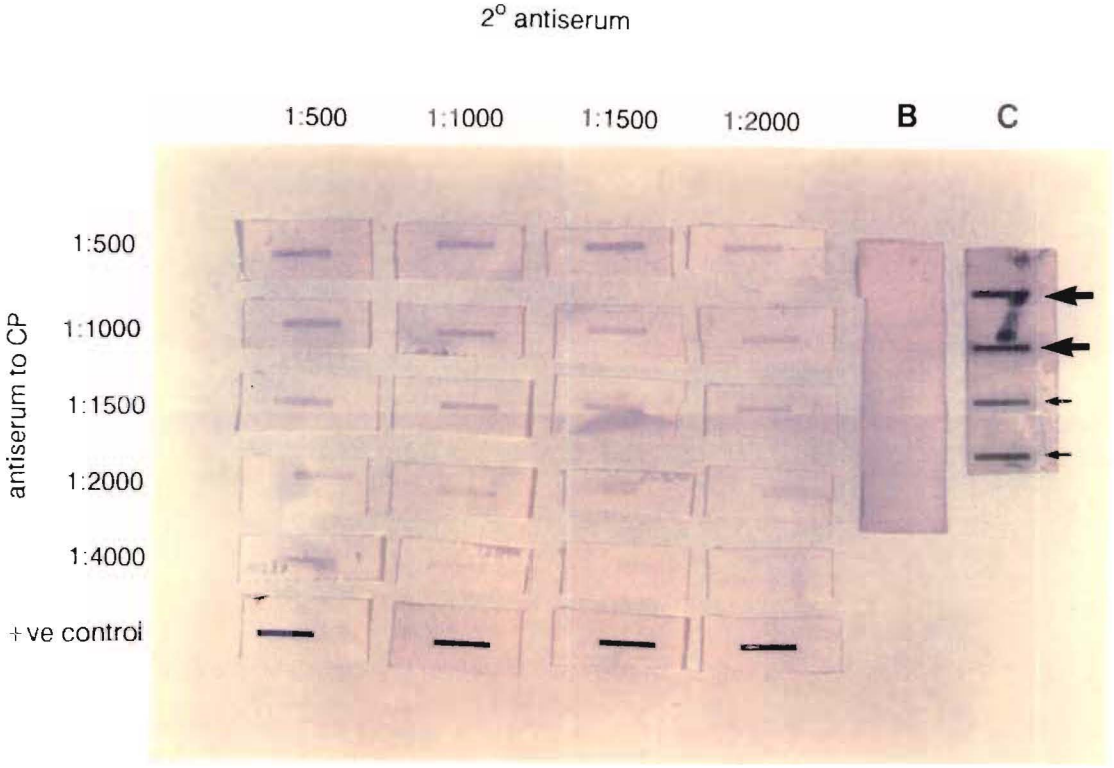


Fig.4.2. A representative protein dot blot using 1° and 2° antisera of different concentrations to determine the optimal concentration of each for use in Western blots. This figure demonstrates the results of using 200 µl infected plant extract incubated with antiserum to PSbMV coat protein as the 1° antiserum. **B.** Uninfected plant control (200 µl). **C.** Plant material (200 µl) reacted with 2° antiserum only (small arrows). Large arrows (lane C) and the +ve control slots comprise 200 ul 2° antiserum (1:500 dilution) applied to the slots followed by incubation with 2° antiserum then substrate.

4.3.2 Optimal Concentration of Primary and Secondary Antisera

Immunodot blots of infected plant extract were used to test the optimal concentration of 1° and 2° antisera. Fig.4.2 shows a representative grid pattern set up to establish the optimal concentrations of antiserum to PSbMV coat protein and 2° antiserum. A similar pattern was observed for each of the other antisera tested. The 2° antiserum (goat anti-rabbit-alkaline phosphatase) was found to give optimal colour development (and by inference optimal binding to the 1° antisera) at a dilution of 1:500. At lower dilutions the colour intensity of the slots was reduced, but it was still evident at 1:2000 dilution. At 1:500 there appeared to be no non-specific binding, as demonstrated by an absence of colour in the uninfected plant controls (lane B). Slots which did not undergo treatment with 1° antiserum (lane C, small arrows) showed the absence of any significant amounts of endogenous alkaline phosphatase in the infected plant material. The slots appear green. The green colour in Lane C is possibly due to the NCM being incubated with only blocking solution followed by 2° antiserum and the washes that normally follow this particular step. As a result of the reduced number of washes and no incubation with 1° antiserum, the green colour (chlorophyll) does not appear to have been removed. The slots indicated by the large arrows in lane C are controls of 2° antiserum (diluted 1:500) applied to the NCM. These slots, which appear almost black, act as controls for the two slots testing for the presence of endogenous alkaline phosphatase (lane C, small arrows). The controls indicate that the reaction of the alkaline phosphatase with the chromogenic substrate is operating satisfactorily. The positive controls shown on Fig.4.2 also consisted of 2° antiserum applied directly to the NCM at a dilution of 1:500. These slots appear black. All 6 1° antisera had good activity down to a dilution of 1:4000, but dilutions up to 1:3000 also had high levels of non-specific binding as shown by the development of colour in uninfected plant material (data not presented). A dilution of 1:4000 was used in all subsequent experiments.

The higher-stringency conditions used for Nla and Al antisera did not give a clearer result. Instead, the slots became fainter and a few were lost (data not presented).

4.3.3 Blocking and Washing Solutions

PBS/Tween plus 3% milk powder had the most effective blocking capacity (observed as low background staining, data not presented). TBS plus BSA was the least efficent, with unacceptably high levels of non-specific binding. It was also apparent that any blocking and washing solution that contained TBS caused the NCM to shrink.

4.3.4 Staining the Markers

Coomassie G250 (Fig.4.3, B) gave the clearest and most consistent results, with the marker bands well defined. The membrane did not alter in size during the staining procedure, thus allowing the molecular weights to be accurately determined. While Coomassie R250 did stain the

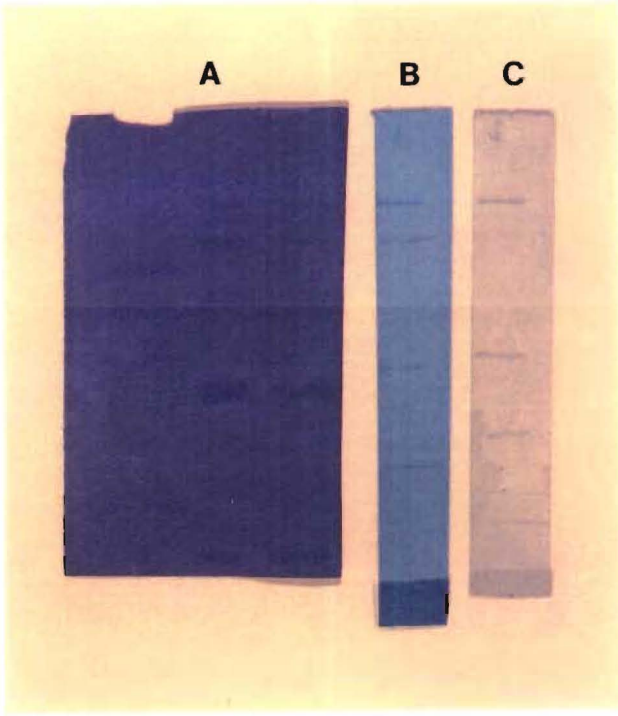


Fig.4.3. A comparison of 3 different stains for visualising molecular weight markers (Biorad). **A.** Coomassie R250. B. Coomassie G250. **C**. Amido black. In this figure the NCM stained with coomassie R250 was from a different gel from either B or C so the length of the NCM cannot be compared. The coomassie G250 and the amido black were from the same gel.

markers, the background colour was frequently so dark that the markers could not be easily distinguished (Fig.4.3, A). Destaining did not solve this problem. The amido black stain had the disadvantage of slightly decreasing the size of the NCM (Fig.4.3, C). This made the estimation of the molecular weights of the specific viral proteins difficult. Note that in this figure the NCM strips stained with Coomassie G250 and amido black were blotted from the same gel, so they were the same length prior to staining. The NCM stained with Coomassie R250, was blotted from a different gel so its length cannot be compared with the other two.

4.3.5 Molecular Weights of the Virus Encoded Proteins

Three of the antisera (TEV NIb, PSbMV CI, and PSbMV CP) showed a high degree of specificity in Western blots (Fig.4.4) as shown by the small number of bands appearing on the NCM. Antiserum to the TEV NIb protein (lane 4) complexed with only a single protein having a molecular weight of 48 kDa. The antiserum to PSbMV CI protein recognised a set of three proteins (lane 5). These had estimated molecular weights of 70, 66, and 64 kDa. The PSbMV CP antiserum (lane 6) complexed with two proteins with estimated molecular weights of 33 and 36 kDa. The molecular weights of the CP and CI proteins to be used in later interpretations of data were chosen mainly by the intensities of the different bands. For the CP, the molecular weight of 33 kDa, decided upon by the band intensities, was confirmed by electrophoresis of purified virus (section 2.3) and by nucleotide sequence data (Appendix A). The "extra" bands are discussed in section 4.4. The uninfected plant material showed no colour for the CP, NIb, and CI antisera (data not presented).

Three antisera (PeMV AI, WMMV AI and TEV NIa) gave ambiguous results in Western blots. The data for PeMV AI are not presented. Both WMMV AI (lane 2) and TEV NIa (lane 3) antisera labelled a number of bands. The WMMV AI antiserum (lane 2) complexed with 4 major bands with molecular weights of 145, 54, 35, and 24 kDa. The TEV NIa antiserum (lane 3) also complexed with 4 bands. The molecular weights of these were 68, 48, 45, and 31 kDa. Faint bands were evident when uninfected plant extract was incubated with WMMV AI or TEV NIa as the 1° antisera. However, these did not correspond to any of the bands labelled in the infected material.

The molecular weights could be clearly identified for three of the PSbMV proteins. These are: NIb (48 kDa); CI (70 kDa); and the CP (33 kDa). For the other two proteins (AI and NIa) Western blots did not provide unequivocal molecular weights. The molecular weight of 54 kDa for the AI protein was decided upon by drawing on published molecular weights for the AIs of other potyviruses. De Mejia *et al.* (1985) reported a molecular weight of 51 kDa for the AIs of PeMV and WMMV-1. A range of 51-56 kDa for the molecular weights of potyviral AIs is cited in Dougherty and Carrington (1988). A band which reacted with the WMMV AI antiserum with an apparent molecular weight of 54 kDa appears to fall into the range outlined for other potyviruses by

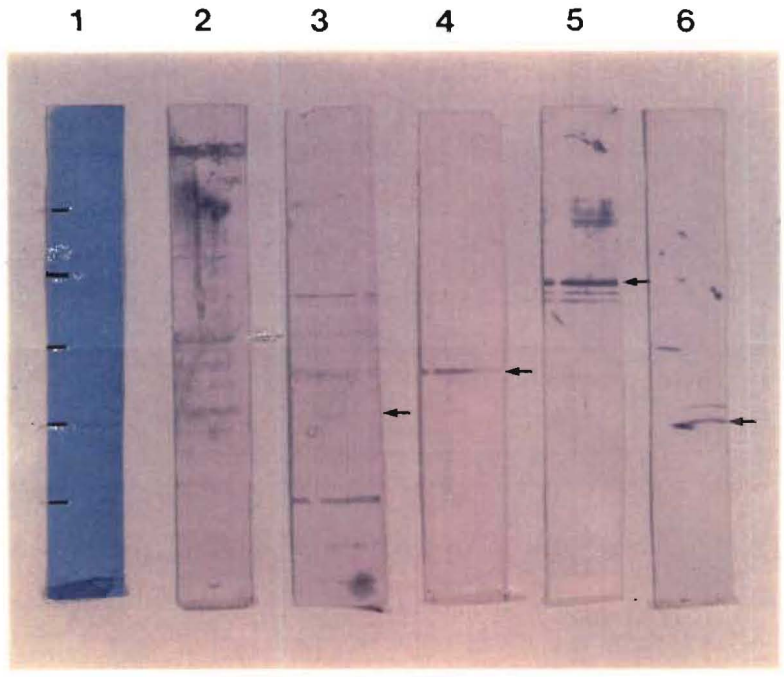


Fig.4.4. A Western blot of total infected pea material reacted with different antisera to determine the molecular weights of specific viral proteins. **Lane 1.** Markers (Biorad). Rabbit muscle phosphorylase b (97,000), BSA (66,200), hen egg white ovalbumin (42,699), bovine carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). **Lane 2.** Primary antiserum to WMMV AI. **Lane 3.** Primary antiserum to TEV NIa. **Lane 4.** Primary antiserum to TEV NIb. **Lane 5.** Primary antiserum to PSbMV CI. **Lane 6.** Primary antiserum to PSbMV CP. The mature proteins are arrowed.

Dougherty and Carrington (1988). Although the size of the Nla protein of PSbMV was ambiguous in the Western blot analysis, immunoprecipitation of *in vitro* translation products with TEV Nla antiserum showed the major band to have a molecular weight of 45 kDa (section 5.3.3). This size corresponds to the predicted molecular weights of one of the bands observed in the Western blot analysis. The molecular weights decided upon for the 5 proteins were: 54 kDa for Al, 45 kDa for Nla, 48 kDa for Nlb, 70 kDa for Cl and 33 kDa for the CP.

4.4 DISCUSSION

The molecular weights of three PSbMV-encoded proteins (CP, Nlb and Cl) were established by Western blot analysis. A molecular weight of 34 kDa for the coat protein of PSbMV (Huttinga 1975) was the only previously established protein size for the virus. This is in close agreement with the 33 kDa established here by Western blot analysis. The molecular weight of 33 kDa for the coat protein is also verified by electrophoresing purified PSbMV (section 2.3) and by nucleotide sequence data (Appendix A). Western blot analysis identified the CP as a doublet having molecular weights of 36 and 33 KDa. The CP appearing as a doublet has previously been reported for several other potyviruses, including BYMV (Hiebert and McDonald 1973), TEV (Hill *et al.* 1973) and PVY (Gough and Shukla 1981). There are several possible explanations for this observation. It may be due to partial degradation of the polypeptide. Shukla and Ward (1988) suggested that removal of the amino and carboxyl termini of the CP may take place when the virus is stored at 4°C. In Chapter 2 and Appendix A, evidence that 53 amino acids at the N-terminus of the coat protein of PSbMV are removed is discussed. It was suggested in Chapter 2 that this may indicate that the coat protein of PSbMV undergoes the type of degradation discussed by Shukla and Ward (1988). However, the doublet band for the CP of PSBMV is evident even when the samples are prepared from fresh plant material. Moreover, Shukla and Ward (1988) discussed the degradation of the potyvirus coat protein mainly in connection with stored virus. An alternative explanation is offered by Dunigan *et al.* (1988). They found the CP of tobacco mosaic virus (TMV) migrated as a doublet in SDS-PAGE with molecular weights corresponding to 17,500 and 26,500 Da. Antibodies to human ubiquitin indicated that the larger protein contained ubiquitinated subunits. Since ubiquitination of proteins is a frequent response to stress, Dunigan *et al.* proposed that this may represent a type of host response to infection. While other explanations such as errors in polyprotein processing cannot be eliminated, the possibility of PSbMV having ubiquinated CP subunits is an attractive one. It must be noted though, that the expected molecular weight gain for a ubiquitinated protein is 8,500 Da but that the difference in the size observed for the two bands reacting with PSbMV CP antiserum is only 3,000 Da. That the "extra" band is larger than the molecular weight determined by SDS-PAGE and nucleotide sequence analysis, and not smaller as would be expected for a degradation product,

suggests material is added to the protein. However, it must not be overlooked that these characteristics are also consistent with alternative processing sites.

The NIb antiserum, although made to protein encoded by TEV, was highly specific with only a single band present on the Western blot. If the NIb is a viral replicase, as Domier *et al.* (1987) proposed, the molecule is likely to have regions of highly conserved sequences. Comparison of the sequence data available for TEV and TVMV indicates a 66.9% degree of identity for the replicases of the two viruses. As such, the recognition of the replicase sequences from other potyviruses is likely. The molecular weight of 48 kDa estimated for NIb of PSbMV is slightly smaller than the NIb of other potyviruses: 54 kDa for BYMV (Chang *et al.* 1988) and 56 kDa for TVMV (Domier *et al.* 1986). However this value is supported by *in vitro* translation in rabbit reticulocyte lysate (see Chapter 5.3).

Antiserum to the CI protein of PSbMV indicated the presence of a set of triple bands. The highest molecular weight band, 70 kDa, labelled the brightest, with the two smaller bands having molecular weights of 66 and 64 kDa. The molecular weight of 70 kDa for the CI protein of PSbMV is similar to weights reported for other potyviruses; for example papaya ring spot virus CI has a molecular weight of 70 kDa (Yeh and Gonsalves 1985). As with the CP, the set of three bands may be due to alternative polyprotein processing sites or proteolytic degradation. The same set of bands is evident in *in vitro* translations of viral RNA in rabbit reticulocyte lysate (see Fig.5.5).

In addition to the three proteins discussed above, estimates of the molecular weights of two other PSbMV-encoded proteins were attempted using Western blots. The molecular weights of neither the AI protein nor the NIa protein were established by Western blot analysis. The antiserum, made against NIa from TEV, demonstrated non-specific binding to plant proteins (using uninfected plant material, data not presented) which was not overcome by increasing the stringency of experimental conditions. The level of homology between proteases from different potyviruses was investigated by Domier *et al.* (1987). Their investigation revealed 48.8% identity between the NIa of TEV and TVMV. Only two clusters of conserved sequences were evident, both at the C-terminus. Despite this, the TEV NIa antiserum successfully immunoprecipitated proteins produced in *in vitro* translations of PSbMV (section 5.3.3). In immunoprecipitations using TEV NIa antiserum the major band, which was unique to immunoprecipitations with this antiserum, had a molecular weight of 45 kDa. This corresponds to one of the bands observed in Western blot analysis. The molecular weight of 45 kDa for PSbMV NIa is slightly lower than the published estimates for other potyviruses- 49 kDa for TEV (Knuhtsen *et al.* 1974) and 52 kDa for TVMV (Hellmann *et al.* 1986).

Hiebert *et al.* (1984) investigated the specificity of AI antiserum to proteins produced by various potyviruses using reciprocal immunoprecipitation experiments. They concluded that the antiserum was specific for a particular virus with very little cross-reactivity between viruses. The uniqueness of this protein may reflect its function in aiding the uptake of a particular virus by a

specific insect vector. However, a number of bands were labelled when either WMMV AI or PeMV AI antisera were used in Western blots. Increasing the stringency of experimental conditions during the period of antigen/antibody recognition did not clarify the results to any appreciable extent. The molecular weight of 54 kDa for PSbMV AI was determined to be the most likely molecular weight for this protein based on published estimates for the AIs of other potyviruses. One of the bands in the Western blots was observed to have this molecular weight.

It had been hoped that with the amount of amplification gained by the use of 2° antiserum conjugated to alkaline phosphatase, it might be possible to detect cross-reactions between unprocessed high molecular weight proteins, as is found with immunoprecipitations from rabbit reticulocyte lysate translations. This was not observed and it can only be assumed that the polyprotein is either never present as an unprocessed molecule or that processing is very rapid and therefore the polyprotein is present for only an extremely short period. As a consequence, only small amounts of unprocessed protein would be present at any one time and these may occur at levels below the detection of the system that was used.

The Western blotting technique gave consistent and reliable estimates of the molecular weights for 3 of the viral-encoded proteins under consideration. The NIa and AI proteins gave less definitive answers. With the exception of the AI protein, all the molecular weights were confirmed by immunoprecipitation in rabbit reticulocyte lysate (see section 5.3.3). The molecular weights of several PSbMV encoded proteins were different in size from the same proteins encoded by other potyviruses.

CHAPTER 5

In vitro TRANSLATIONS OF PSBMV RNA IN RABBIT RETICULOCYTE LYSATE

5.1 INTRODUCTION

At the initiation of this study, the translation strategy of the potyviruses and the organisation of their genome was still the subject of debate. Over the past decade, a number of expression strategies for the various members of the group have been proposed.The first translations of plant viral RNA in messenger dependent lysate (MDL) used TMV (Pelham and Jackson 1976), followed by cowpea mosaic virus RNA (Pelham 1979), TEV (Koziel *et al.* 1980), PeMV and TEV (Dougherty and Hiebert 1980a,b,c) and TVMV (Hellmann *et al.* 1986). From early cell-free translations of PeMV and TEV, Dougherty and Hiebert (1980c) were able to propose a gene map for these two viruses. Their map showed the nuclear inclusion proteins separated by two genes, one coding for the cylindrical inclusion protein and the other for the 41-50 kDa protein. Allison *et al.* (1986) and Carrington and Dougherty (1987b) revised the map, placing the two nuclear inclusion genes adjacent to one another. Hellmann *et al.* (1986) proposed a similar genomic organisation for TVMV. This organisation suggested a gene cluster composed of CI,VPg,NIa,NIb, which is also found in two other groups of viruses, the picornaviruses and the comoviruses. These two groups are considered to be similar to potyviruses in their organisation (Argos *et al.* 1984; Domier *et al.* 1987).

Originally there was some confusion over whether or not the coat protein gene was encoded on a subgenomic RNA (sgRNA). Otal and Hari (1983), working with TEV RNA isolated from plant material, proposed the presence of seven sgRNAs for this virus. This strategy is used by a number of plant viruses, especially to encode the coat protein. For example, in comoviruses the structural proteins are encoded by a second RNA that is not required for virus replication (Rezelman *et al.* 1980). Amplification of the translation product of the coat protein gene is an understandable strategy since approximately 2,000 coat protein monomers are required to coat each potyviral RNA molecule (Hollings and Brunt 1981). However, the presence of sgRNAs for TEV was disproved by Dougherty (1983). Subsequently the question of sgRNAs was reopened by Dougherty *et al.* (1985) after they analysed the nucleotide sequence of pepper mottle virus (PeMV). They suggested the coat protein of this virus was translated either from an internal initiation site or from a sgRNA.

The position of the coat protein gene has also been the subject of debate. Vance and Beachy (1984a) suggested the coat protein gene was located near the 5' end of the molecule. This deduction arose from the observation that the major *in vitro* products of SMV-RNA were

polyproteins related to the coat protein. Hellmann *et al.* (1980) suggested a similar arrangement for TVMV. However, Dougherty and Hiebert (1980c) proposed gene maps for TEV and PeMV which placed the coat protein at the 3' end of the viral genome. This position has subsequently been confirmed for a number of other potyviruses, including PSbMV (by nucleotide sequence analysis, Appendix A). The genomic organisation for both TEV and TVMV proposed by Dougherty and Carrington (1988), and the proposed functions for the various gene products is presented in Fig.5.1 and Table 5.1, respectively. It is noted that these authors have ignored the possibility that the 70 kDa protein is involved in cell-to-cell spread, as proposed by Langenberg (1986). Evidence presented in Chapter 3 suggests that the 70 kDa protein may have multiple functions.

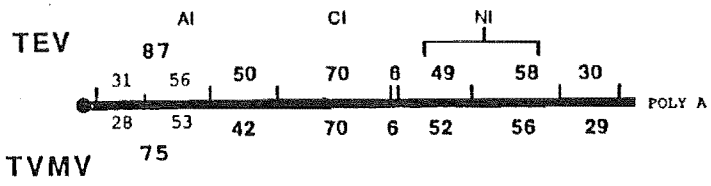


Fig.5.1. A schematic drawing comparing the genomic organisation of TEV and TVMV RNAs. The molecular weights (x 10^3) of the putative cleavage products are present of each virus. The • represents the position of the VPg. (Taken from Dougherty and Carrington 1988).

TEV GENE PRODUCT	PUTATIVE FUNCTION
31 kDa	Cell-to-cell movement
56 kDa	Insect Transmission
50 kDa	Polyprotein Processing (Protease)
70 kDa	Replication
6 kDa	Replication
49 kDa	Polyprotein processing (Protease)
58 kDa	Replication (RNA-Dependent RNA-Polymerase)
30 kDa	Encapsidation

Table 5.1. A list of the putative functions attributed to the products of TEV by Dougherty and Carrington. The molecular weights of the products are given in kDa. (Taken from Dougherty and Carrington 1988).

The nucleotide sequence analyses of TEV- and TVMV-RNAs by Allison *et al.* (1986) and Hellmann *et al.* (1986) confirmed the presence of a single open reading frame capable of coding for polyproteins of 346 kDa and 340 kDa respectively. The analyses support the evidence from translations that a single polyprotein is synthesised and subsequently cleaved by virus-encoded proteases (Carrington and Dougherty 1987b; Hellmann *et al.* 1988). More recently, a second virus-encoded protease has been confirmed by Carrington *et al.* (1989) using cell-free expression of defined TEV-RNA transcripts. This protease, like Nla, appears to cleave by an autocatalytic

mechanism. The carboxyl-terminal 20 kDa domain within the 87 kDa protein of TEV (possibly corresponding to the 86 kDa protein of PSbMV, see section 5.3.2) is required for proteolysis (Carrington *et al.* 1989). Their conclusion is of considerable interest, as Thornbury *et al.* (1985) have proposed that this protein is the helper component in PVY and TVMV. Carrington *et al.* (1989) suggested that this protein has two active sites, one at the carboxyl terminus for proteolytic cleavage, and one at the amino terminus for helper component activity. A third protease may be present, because the 49 kDa protease and the newly discovered protease discussed above do not together account for all the cleavages necessary to produce the mature proteins observed. Alternatively, auxillary factors may complement one of the proteases to facilitate the additional cleavages (Carrington *et al.* 1989).

The functions of several other features of the RNAs of TEV and TVMV remain obscure. Both TEV and TVMV RNAs have an untranslated region at the 5' end of the genome. Sleat *et al.* (1987) suggested a function for the 5' leader sequence of TMV RNA as a general enhancer of translation. In a later paper, Sleat *et al.* (1988) observed that the 5' leader sequence from brome mosaic virus RNA3, alfalfa mosaic virus RNA4 and the genomic RNAs of the tomato strain of tobacco mosaic virus, Rous sarcoma virus and TMV enhanced mRNA translation in eukaryotic systems. It has been demonstrated that RNA sequences present upstream and downstream of a reporter gene coding region play an important role in determining the amount of protein produced from a mRNA (Gallie *et al.* 1989). A translational enhancer derived from TMV increased the efficiency of translation of a reporter gene by 16-18 fold in electroporated protoplasts.

Several potyviruses are known to possess polyadenylated RNAs (Hari *et al.* 1979; Hay *et al.* 1989). A minimum poly[A] tail of 25 adenylate residues was reported by Gallie *et al.* (1989) to be sufficient to substantially increase the expression and half-life of a reporter mRNA in plants. It has been noted that all mRNAs examined so far terminate in a poly[A] tail, with the exception of those coding for cell-cycle regulated histones (reviewed in Birnstiel *et al.* 1985). In *Dictyostelium*, mRNAs with poly[A] tails longer than 30 residues are recruited more efficiently onto polysomes and are thereby expressed more frequently (Shapiro *et al.* 1988). It has been suggested that the mechanism of enhancement rests on slowing the action of the 3'-5' exonucleases. Other mechanisms cannot be eliminated, however (see Brawerman 1981). Gallie *et al.* (1989) also observed that a 5' untranslated region and a poly[A] tail function independently of each other, but that their combined effect on translation is multiplicative.

In addition to the presence of a 5' untranslated region and a poly[A] tail, members of the potyvirus family have an untranslated region at the 3' end of the viral genome (Hay *et al.* 1989; Domier *et al.* 1986; Allison *et al.* 1986). The function of the untranslated region is not known. However, the 3' and 5' regions of the potyvirus RNA probably have a role in replication. The VPg, found covalently attached to the 5' end of the genome, also probably has a role in replication (Dougherty and Carrington 1988).

In the current chapter, a search for a sgRNA for the coat protein gene of PSbMV was undertaken. RNA extracted from both polyribosomes of infected plants and total plant RNA has been analysed by Northern blots. In addition, the organisation of the PSbMV genome is also examined using *in vitro* translation in rabbit reticulocyte lysate and the immunoprecipitation of viral products. Finally a gene map for PSbMV is proposed.

5.2 MATERIALS AND METHODS

Preparation of Materials for Northern Blots

5.2.1 Isolation of Polyribosomal RNA

The polyribosomal RNA isolation procedure was modified from the methods of Jackson and Larkins (1976) and Vance and Beachy (1984a). All glassware, plasticware and other vessels and solutions used in these experiments were RNase-free (see Chapter 2.2).

Samples of infected or uninfected pea leaf (0.6 g) were ground in liquid nitrogen in a mortar and pestle with 6 ml of extraction buffer containing 200 mM Tris-HCl pH 9.0, 400 mM KCl, 200 mM sucrose, 35 mM $MgCl_2$, 25 mM ethylene glycol bis tetra acetic acid (EGTA) pH 9.0, 100 μg ml^{-1} cyclohexamide and 0.5% Triton X-100. The sample was centrifuged at 15,500 rpm for 10 minutes in a Sorvall SS-34 rotor. The supernatant was layered onto a 3.4 ml sucrose cushion containing 1.75 M sucrose, 40 mM Tris-HCl pH 9.0, 200 mM KCl, 30 mM $MgCl_2$ and 5 mM EGTA. The ribonucleoprotein particles (RNP) were pelleted at 41,000 rpm for 66 minutes in an SW 41 rotor (Beckman). The pelleted RNPs were suspended in 5 ml of buffer containing 40 mM Tris-HCl pH 8.5, 4 mM EDTA, 200 mM KCl and 30 mM $MgCl_2$ and held on ice for 10 minutes to dissolve the polyribosomes (Blobel 1971). The sample was loaded onto a second sucrose cushion and centrifuged as before. The RNP fraction was suspended in an equal volume (200 μl) of isolation buffer containing 200 mM Tris-HCl pH 9.0, 2% (w/v) SDS, 25 mM EDTA, 300 mM NaCl and 200 μg ml^{-1} predigested proteinase K. This was mixed thoroughly and incubated at 37°C for 30 minutes. The RNA was separated from the protein by phenol/chloroform extraction and collected by ethanol precipitation. An aliquot of RNA was treated with 2 M lithium chloride (LiCl) to remove the tRNA and DNA as described by Palmiter (1974). One ml of 2 M LiCl in 25 mM NaOAc pH 5.0, was added to the RNA and vortexed gently. The tube was incubated on ice for 10-20 minutes, and centrifuged at 27,000 g for 10 minutes. This process was repeated once. The pellets were dissolved in 2 ml 0.1 M NaOAc pH 7.0 and the RNA collected by ethanol precipitation, dried and resuspended in 5 μl 25 mM EDTA and 0.1% SDS. The concentration of RNA was determined spectrophotometrically (Maniatis *et al.* 1982). An aliquot was analysed on a 1% agarose gel in 1 x TBE to determine the size and state of degradation of the RNA.

5.2.2 Isolation of Total Plant RNA

Total plant RNA from infected and uninfected plants was isolated by the method of Habili *et al.* (1987). Plant tissue (0.1 g) was homogenised in a glass tissue grinder in 1 ml of buffer (0.5 M NaOAc pH 6.0, 10 mM $MgCl_2$, 3% (w/v) SDS and 20% ethanol) and 1 ml of buffer saturated phenol. The sample was centrifuged at 5,000 g for 10 minutes. The supernatant was washed with an equal volume of chloroform:isoamyl alcohol (24:1). The phases were separated by centrifugation at 5,000 g for 5 minutes and the aqueous phase was collected. The RNA was ethanol precipitated and the dried pellet was resuspended in 100 μl TE. The concentration of the RNA was determined spectrophotometrically (Maniatis *et al.* 1982). An aliquot was analysed on a 1% agarose gel in 1 x TBE and the electrophoretic mobilities of the components were compared to the 1 kb DNA ladder (BRL).

5.2.3 cDNA Probe Preparation

A probe for Northern blotting was made from the clone pPSb70 (see section 2.2.5 for preparation of the clones). Sequence data have shown pPSb70 to encode the entire coat protein gene, plus a small fraction of the adjacent 5' gene (Appendix A). A scaled-up alkaline mini preparation (section 2.2.9) was used to prepare large quantities of pUC19 containing the insert (pPSb70), which was then excised from pUC19 using the restriction endonucleases *BamH*I and *Eco*RI. The insert was separated from pUC19 on a preparative 6% polyacrylamide gel as described in section 2.2.5. The resulting DNA fragment was resuspended in dH_2O and stored at 4°C. Using a Multiprime Kit (Amersham), 25 ng of cDNA was labelled with ^{32}P.

NORTHERN BLOT

The Northern blot procedure was modified from the methods described by Fourney *et al.* (1988) and given in the Gene Screen Plus manufacturer's manual.

5.2.4 Sample Preparation and Transfer of RNA to Membrane

Between 80 and 135 μg of RNA was prepared for electrophoresis by adding 5 μl of 25 mM EDTA pH 7.2 and 0.1% SDS (w/v) to the RNA pellets. To this, 25 μl of electrophoresis sample buffer was added. The buffer contained 0.75 ml deionised formamide, 0.15 ml 10 x MOPS [0.2 M 3-(N-morpholino propanesulfonic acid), 50 mM NaOAc, 10 mM EDTA adjusted to pH 7.0], 0.24 ml formaldehyde, 0.1 ml dH_2O, 0.1 ml glycerol and 0.08 ml 10% (w/v) bromophenol blue. The sample was heated at 65°C for 15 minutes and cooled on ice. One μl of 1 mg ml^{-1} ethidium bromide was added to each sample and mixed thoroughly. Reconstitution experiments in which 1 μg of viral RNA was added to uninfected plant material prior to the isolation of total RNA, or 10

pg of viral RNA was added to total RNA isolated from uninfected plants were conducted. Positive controls of 10 pg probe or 10 pg purified viral RNA were also included.

The total sample was loaded onto a 1% (w/v) agarose gel in 1 x MOPS containing 0.66 M formaldehyde. The 6 mm thick gel was allowed to solidify for one hour before loading. The electrophoresis buffer consisted of 1x MOPS buffer and the gel was electrophoresed at 30 V at room temperature for 18 hours. Following electrophoresis, the gel was inspected using a short wave ultraviolet light transilluminator. The gel was prepared for transfer to a nylon membrane (Gene Screen Plus) by soaking it in 10 x SSC (1.5 M NaCl and 0.15 M Na citrate) for two 20 minute periods. The RNA was transferred to the membrane by capillary action. The blot was assembled according to the Gene Screen manufacturer's instructions.

After transfer, the gel was examined on the transilluminator to establish the efficiency of transfer. The membrane was rinsed in 2 x SSC to remove residual agarose and allowed to dry at room temperature. It was then baked at 80°C for two hours to reverse the formaldehyde reaction.

5.2.5 Prehybridisation and Hybridisation of the Membrane

The membrane was placed in a bag containing 0.1 ml of prehybridising buffer per cm^2 membrane. The prehybridisation buffer consisted of 50% deionised formamide, 6 x SSC, 1% SDS, 10% dextran sulphate and 30 μg per ml denatured herring sperm DNA. The bag was agitated at 42°C for 4 hours. 9.3 ng of radioactive probe (prepared as in section 5.2.3) was denatured by heating at 100°C for two minutes, added to two ml of fresh prehybridisation buffer, and then added to the solution in the bag. A further 30 μg of denatured herring sperm DNA was also added. The final volume of the solution in the bag was 10 ml. Hybridisation continued for 12 hours at 42°C with constant agitation.

5.2.6 Washing Procedure

The membrane was removed from the hybridisation solution and washed with constant agitation as follows: two washes in 100 ml of 2 x SSC at room temperature, two further washes in 200 ml of 2 x SSC and 0.1% SDS at 60°C for 30 minutes, and two in 100 ml of 0.1 x SSC at room temperature for 30 minutes.

The membrane was then sealed in a plastic bag and exposed to Cronex 4 X-ray film (Dupont) at -80°C using intensifying screens.

TRANSLATION IN RABBIT RETICULOCYTE LYSATE

5.2.7 Standard Reaction

Messenger-dependent rabbit reticulocyte lysate (MDL) was kindly donated by R. Forster, Plant Diseases Division, DSIR, Auckland. The standard protein synthesis mixture comprised 10 μl of MDL containing 10 mM creatine phosphate, 8 μCi [^{35}S] methionine (500-800 Ci/mmol) and 25-150 μM of 19 L-amino acids, omitting methionine (Jackson and Hunt 1983). The final concentrations of the reducing agents were: 0.5 mM DTT (from the unlabelled amino acid stock solution) and 0.5 mM β mercaptoethanol (from the [^{35}S] methionine).

Final cation concentrations were optimised for the quality and quantity of the ^{35}S labelled products from the translation of PSbMV RNA. These concentrations were determined by testing the following permutations: 2KM, KM, 0.5K M, 1.5K M, K-M, K 0.5M, and K 1.5M where K equals 2 M KOAc and M equals 10 mM MgOAc. 1.1 μl of the appropriate KM permutation was added to the translation mix. 220 mM K^+ and 1.1 mM Mg^{2+} gave the optimal results (see section 5.3.2).

All reactions contained one μg PSbMV RNA, except for controls where the RNA was replaced by the appropriate volume of dH_2O. All incubations were at 37°C for 50 minutes unless stated otherwise.

5.2.8 Addition of Putative Inhibitors of Polyprotein Processing

Analogues

MDL, lacking the amino acids arginine, lysine and phenylalanine, was supplemented with their respective analogues L-canavanine (final concentration 5 μM), N-tosyl-lysyl-chloromethane (20 μM) and *p*-fluorophenylalanine (15 μM).

Antiserum to the 49 kDa NIa of TEV (Putative Protease)

A standard reaction was set up, and 1.5 μl of antiserum to the TEV 49 kDa NIa protein was added to this. Incubation times varied from two minutes to 50 minutes (standard time). In a second experiment, the antiserum to the TEV 49 kDa NIa protein was added to MDL containing the amino acid analogues and incubated for 30 or 50 minutes. Pre-immune rabbit serum and antiserum to the TEV 54 kDa NIb protein were used as controls for both experiments.

5.2.9 Addition of Dithiothreitol (DTT)

DTT was added to the standard reaction mixture to a final concentration of 5 mM and incubated under the standard conditions already outlined.

5.2.10 Time Course Experiment

A standard reaction mixture was incubated for 2, 6, 10, 20, 30, and 50 minutes.

5.2.11 Electrophoresis of Samples

20 μl of SDS sample loading buffer (Laemmli 1970) was added to the samples and mixed thoroughly. 10 μl was loaded onto a 10 ml 10% (w/v) polyacrylamide gel containing 0.1% SDS, 0.1% APS and 12.5 μl TEMED in 0.37 M Tris-HCl pH 8.8. The 5 ml stacking gel contained 3% acrylamide, 0.1% SDS, 0.04% APS and 7.5 μl TEMED in 0.125 M Tris-HCl pH 6.8. The gel was 0.75 mm thick. 20 μl of prestained protein molecular weight marker standards (BRL) were loaded according to the manufacturers instructions. The electrophoresis buffer contained 300 mM glycine, 50 mM Tris base and 0.1% SDS. ^{35}S- labelled proteins were separated at 3 mA/cm until the dye front reached the bottom of the gel. Gels were fixed in 7% glacial acetic acid for 3 minutes, drained briefly, then agitated in 'Amplify' (Amersham) according to the manufacturer's instructions. The gels were dried on Whatman 3MM filter paper and the radiolabelled polypeptides were detected by fluorography on Cronex 4 X-ray film (Dupont) exposed at -80°C overnight using intensifying screens.

IMMUNOPRECIPITATION OF VIRAL POLYPEPTIDES USING ANTISERA TO THE PSBMV CP, TEV NI$_a$, TEV NI$_b$, PSBMV CI AND WMMV AI PROTEINS

5.2.12 Standard Reaction

The standard reaction in which the translation products were synthesised prior to immunoprecipitation had a final volume of 16 μl. The components were in the same final concentrations as stated for the translation experiments (section 5.2.7) except that 15 μCi [^{35}S] methionine was used. Incubation of the samples was for 50 minutes at 37°C unless stated otherwise. After this period, 8 μl of pre-immune rabbit serum (gifts from R. Forster, Plant Diseases Division, DSIR, Auckland and J. Lewis, Haematology Department, Christchurch Hospital, Christchurch), 60 μl 1 M NaCl/1% Triton X-100 and 60 μl of freshly prepared protein A Sepharose (Sigma, 62.5 mg ml^{-1} in 25 mM Tris-HCl pH 7.5) were added to the sample and incubated at 4°C for two hours with gentle mixing. The immunoadsorbants were recovered by centrifugation at 8,000 g for two minutes. The pellets were washed 6 times with 60 μl 0.75 M NaCl/1% Triton X-100 and the supernatant fraction and the washings pooled. Initially 8 μl of each antiserum was added to a tube of supernatant. This was reviewed after analysis of the initial results. Subsequently different amounts of sera were added depending on the apparent activity of the particular antiserum. Final volumes were: 6 μl of PSbMV CP and TEV NIb, 7 μl PSbMV CI, 8 μl TEV NIa and 10 μl AI (for both WMMV and PeMV anti-AI). For each tube the appropriate volume of the

particular antiserum was added together with 60 μl of 62.5 mg ml^{-1} protein A Sepharose and gently mixed overnight at 4°C. The immunoadsorbants were washed and collected as before. The pellet was resuspended in 20 μl 2 x Laemmli sample buffer (Laemmli 1970), heated at 100°C for two minutes to release the bound proteins and cooled on ice. The sample was centrifuged at 8,000 g for 5 minutes and the supernatant was collected. A second 10 μl aliquot of 2 x sample buffer was added to the pellet, mixed thoroughly and centrifuged. The two supernatants were pooled and loaded onto a 10% polyacrylamide gel as described in section 5.2.11. The gel was treated in the same manner as the translation gels above, except that they were exposed to the X-ray film for 3 days.

5.2.13 Addition of Putative Inhibitors of Polyprotein Processing

Several experiments parallel to those conducted for the translations were conducted. In the first experiment, translation was carried out in the presence of the TEV 49 kDa Nla antiserum. Two μl of TEV 49 kDa Nla antiserum was added to the 16 μl standard immunoprecipitation translation reaction and incubated for the standard time. Controls containing pre-immune rabbit serum were included. In a second experiment, proteins were translated in lysate containing the amino acid analogues to arginine, lysine and phenylalanine. The radiolabelled proteins were immunoprecipitated with antiserum to the TEV Nlb protein only. Thirdly, a time course experiment was conducted in which translation was stopped after 5, 10, and 15 minutes by the addition of 1 μg RNase. The products of these translations were immunoprecipitated with antiserum to the TEV Nlb protein. The immunoadsorbants were collected and electrophoresed as described above. Immunoprecipitated proteins were detected by fluorography.

5.3 RESULTS

5.3.1 Northern Blots

A Northern blot of RNA isolated from the polyribosomes of infected pea leaves showed the presence of a band at 10 kb (Fig.5.2 Lane 3). This corresponds to the approximate molecular weight of full length PSbMV RNA. A lot of smaller reacting material is also present in this lane. The smaller material is possibly the result of the degradation of full length PSbMV RNA by RNases endogenous to plant material. Alternatively, the RNA may have undergone breakage during the isolation of the polyribosomes and subsequent dissociation of the RNA from the polysomes. The former explanation appears more likely and is supported by the results of the reconstitution experiments (Fig.5.3).

Autoradiographs of Northerns blots were examined for the presence of a band that could represent a sgRNA for the CP of PSbMV. An autoradiograph comparable to that seen in Fig.5.2

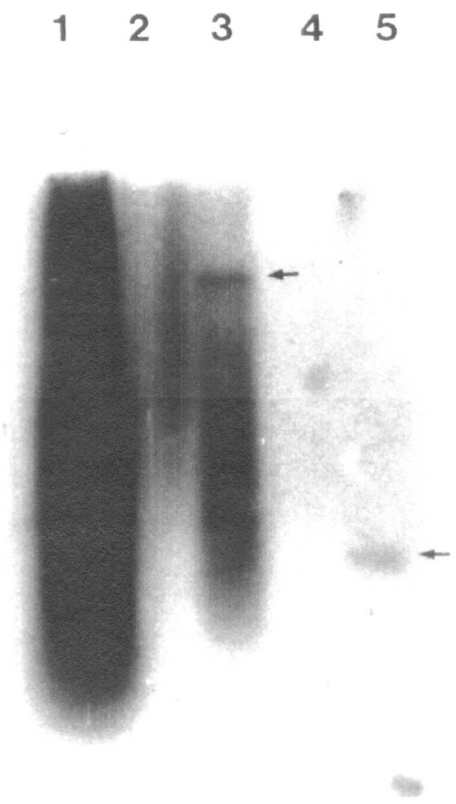


Fig.5.2. Northern blot using ^{32}P-labelled pPSb70 probe. **Lane 1.** Total RNA from an infected plant (134 μg). **Lane 2.** Total RNA from an uninfected plant (133 μg). **Lane 3.** RNA isolated from polysomes of an infected plant (40 μg). The arrowed band indicates a 10,000 nucleotides RNA species. **Lane 4.** RNA isolated from polysomes of an uninfected plant (80 μg). **Lane 5.** 10 pg of probe (arrowed). This corresponds to approximately 1,200 bp.

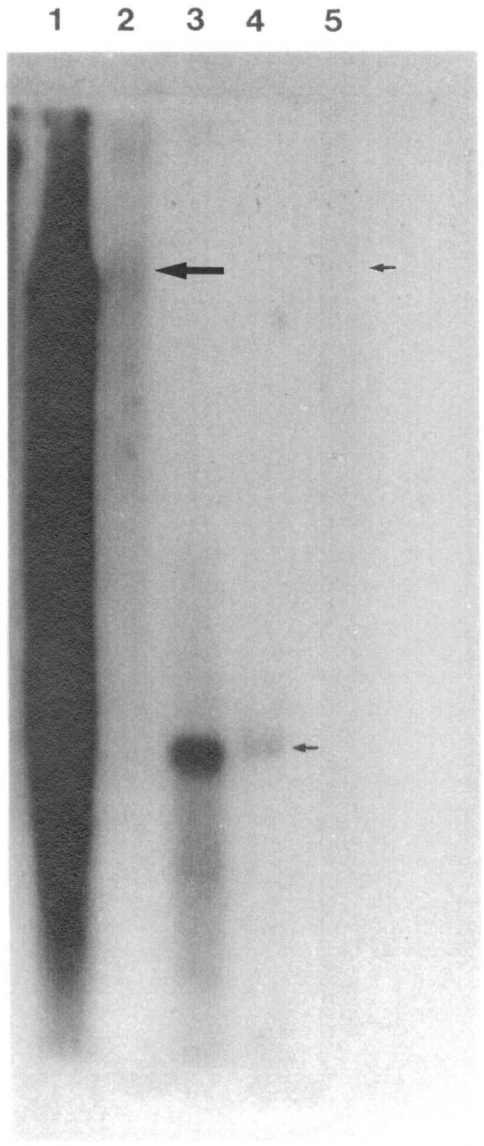


Fig.5.3. Northern blot of the reconstitution experiment. **Lane 1.** 5 μl of uninfected plant homogenate plus 1 μg PSbMV-RNA added before the preparation of the RNA. **Lane 2.** 200 μg of total uninfected plant RNA plus 10 pg of PSbMV-RNA added after preparation of the RNA. **Lane 3.** 10 pg of probe (pPSb70) (arrowed). **Lane 4.** 1 pg probe. The probe is approximately 1,200 bp. **Lane 5.** 1 kb ladder (BRL). The position of the 10,000 bp band from the 1 kb ladder (BRL) is arrowed.

but exposed for only 24 hours instead of the 96 hour exposure of the autoradiograph seen in Fig 5.2 was examined for the presence of a sgRNA for the CP. In the shorter exposed autoradiograph the band corresponding to full length RNA (lane 3) could still be seen but the lighter intensity of the autoradiograph allowed some conclusions to be drawn regarding the presence of a sgRNA for the CP gene of PSbMV. No band corresponding to the molecular weight of a sgRNA was observed in lane 3. Lane 3 contained RNA isolated from the polysomes of an infected plant, which indicates that a sgRNA for the CP is not actively being translated. The positive control (Lane 5) is approximately 1,200 bp and is slightly larger than the gene encoding the coat protein of PSbMV. (The probe represents the coat protein gene plus part of the upstream gene).

The total plant RNA isolated from an infected plant was observed to hybridise with the DNA probe, resulting in a smear (Fig.5.2 lane 1). Some of the smear that is observed in lane 1 may be due to the probe binding non-specifically to the plant material. This is substantiated by the presence of a smear in lane 2, where although the RNA from lane 2 was derived from uninfected plant material a smear can still be seen. This is presumably due to non-specific binding to plant material. The total amount of RNA loaded onto the gel was similar in both lanes 1 and 2 (Lane 1 134 μg; Lane 2 133 μg), but a significantly darker smear is observed in lane 1, indicating that non-specific binding to plant material can not alone account for the smear. The rest of the smear observed in Fig.5.2 lane 1 would appear to be due to the probe reacting with a spectrum of different sized fragments of PSbMV RNA. The polysomal RNA from uninfected plant material (Fig.5.2 lane 4) did not hybridise with the probe.

In the reconstitution experiments (Fig.5.3), purified PSbMV RNA was added both before the isolation of total RNA from an uninfected plant (Fig.5.3 Lane 1) and after the RNA was isolated from an uninfected plant (Fig.5.3 Lane 2). In both cases, the viral RNA has undergone degradation or breakage producing a lot of smaller material reacting with the probe. Because PSbMV RNA added after the isolation of total plant RNA still showed smaller material reacting with the probe (Fig.5.3 lane 2), degradation seems the more likely explanation.

In Fig.5.3, a band corresponding to 10 kb appears to be present in lane 2 (large arrow). This corresponds to the approximate size of the full length RNA that was added. However, it may also be bleed-over from lane 1 which is overloaded.

5.3.2 Translation in Rabbit Reticulocyte Lysate

The quality and quantity of PSbMV-RNA encoded polypeptides produced in response to the varying K^+ concentrations is seen in Fig.5.4. The ratio, KM (corresponding to 220 mM K^+ and 1.1 mM Mg^{2+}), provided optimal translation conditions for PSbMV-RNA (Fig.5.4 lane 4) and was used in all following translations. In translations where other concentrations of K were used (Lanes 3, 5, and 6), the quantity of translation products appeared to be lower (estimated by comparing the intensity of the bands). The quality was also lower, with some bands absent in

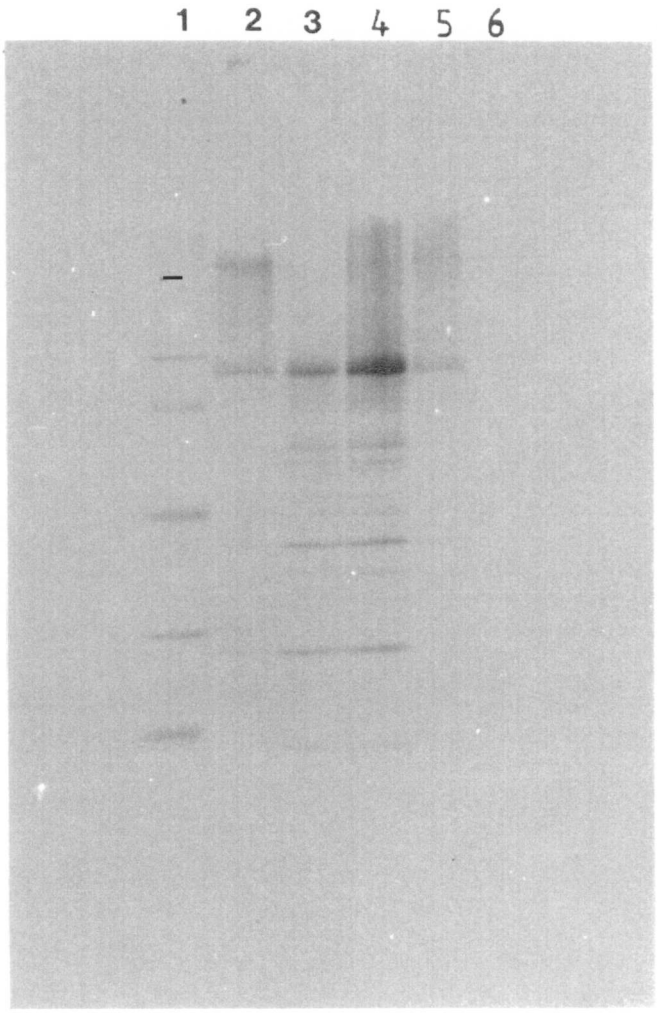


Fig.5.4. Comparison of the translation products of PSbMV-RNA at varying cation concentrations and the effect of including DTT in the translation mix. **Lane 1.** Radiolabelled markers (Amersham) (x 10^3 Da). Myosin (200), phosphorylase b (92.5), BSA (69), ovalbumin (46), carbonic anhydrase (30), trypsin inhibitor (21.5) and lysozyme (14.3). **Lane 2.** DTT included. **Lane 3.** 0.5K M. **Lane 4.** KM. **Lane 5**. 1.5K M. **Lane 6**. 2K M.

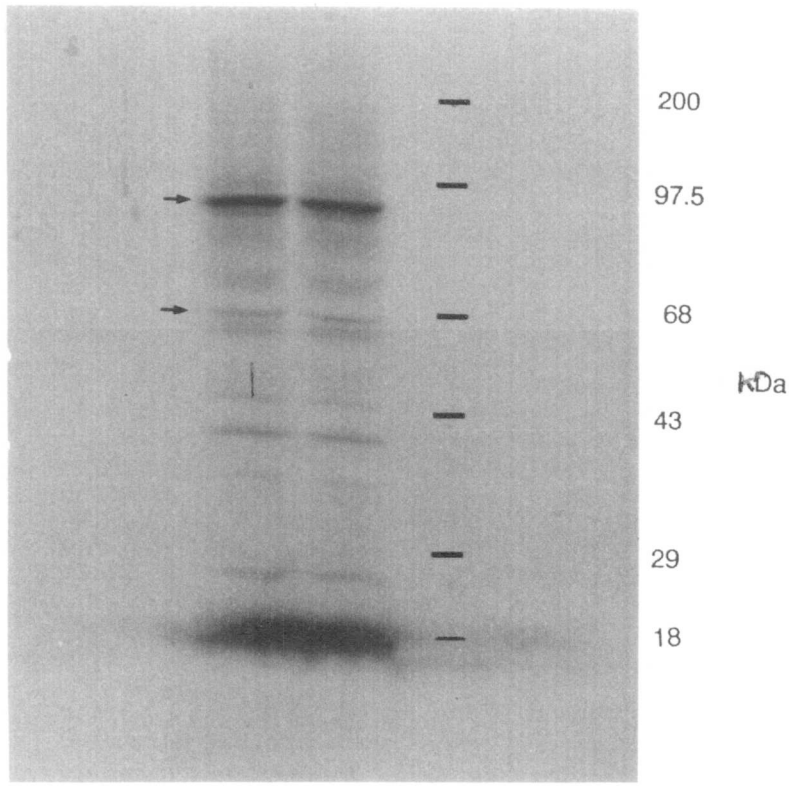


Fig.5.5. Total translation products of PSbMV-RNA. The major band with an estimated molecular weight of 86 kDa is arrowed. A set of triple bands whose mobility correspond to products related to the CI protein are arrowed. The molecular weights of the markers (BRL prestained markers) (x 10^3 Da) are as follows: myosin (200), phosphorylase b (97.5), BSA (68), ovalbumin (43), carbonic anhydrase (29) and B lactoglobulin (18).

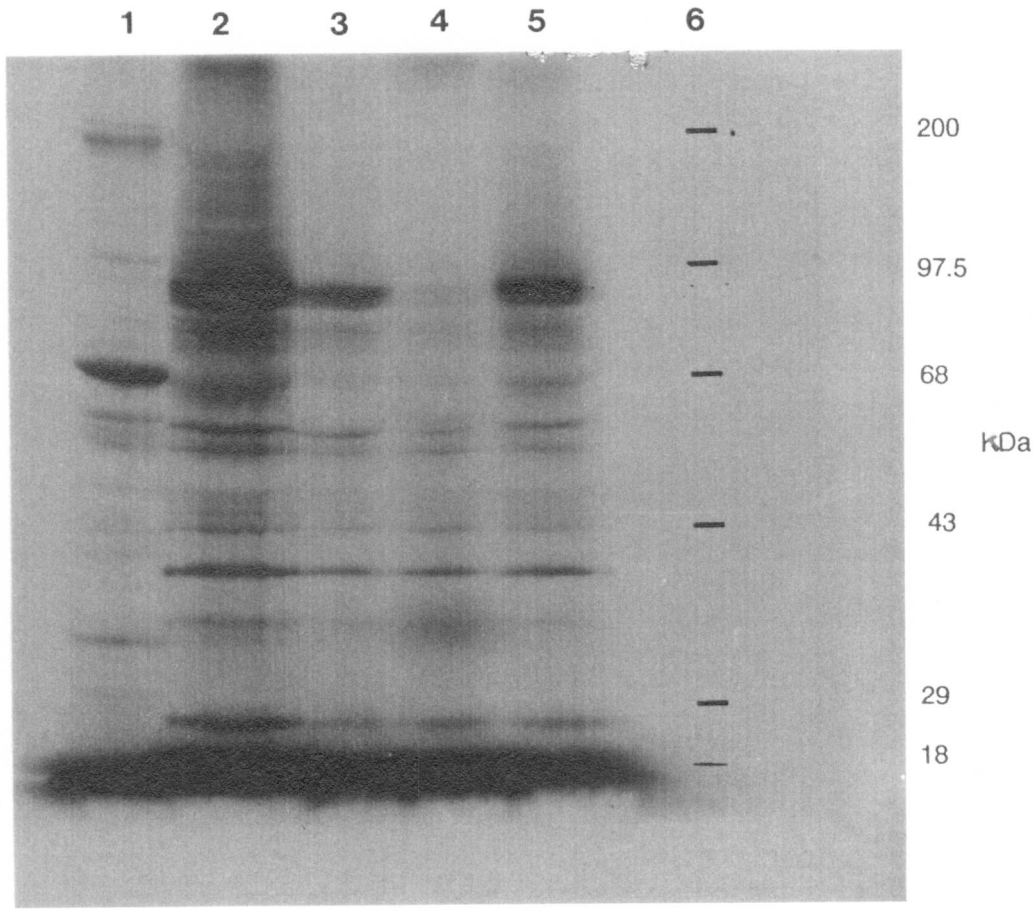


Fig. 5.7. The effect of including antiserum to the 49 KDa protein or amino acid analogues in the translation mix on the size of the polypeptides accumulating in MDL. **Lane 1.** H_2O control. **Lane 2.** Standard translation. **Lanes 3-5.** Translations carried out in the presence of; **3**. Antiserum to 49 KDa protein; **4**. Pre-immune rabbit serum; **5**. Amino acid analogues. **Lane 6.** Markers (the same as in Fig.5.5.)

some lanes. A similar experiment established the optimal concentrationof Mg^{+2} (data not presented).

Fig.5.4 lane 4 demonstrates the broad size range of PSbMV RNA-encoded polypeptides, from 238 kDa to 18 kDa. More than 11 major species of protein were observed on the original fluorograph. Six of the major proteins observed had molecular weights greater than any single mature protein. The molecular weights of these 6 polypeptides were approximately 238, 196, 157, 115, 112, and 86 kDa. Fig.5.5 shows more clearly the major band with a molecular weight of 86 kDa (top arrow). The 86 kDa band appears to be a doublet and will be discussed in section 5.3.3. A set of triple bands (lower arrow) can be seen which have the same mobility as the triple bands reacting with antiserum to the PSbMV CI protein in Chapter 4 Fig 4.4. In addition, bands with molecular weights of 48, 45, and 33 kDa were observed.

Polyprotein Processing Inhibitors

Several different treatments (inclusion of amino acid analogues, inclusion of antiserum to the putative protease, or adding additional DTT to the translation mix) were tried in an attempt to inhibit the processing of the polyprotein. None of the treatments was successful.

Inclusion of the amino acid analogues in the translation mixture (Fig.5.7 lane 5) had no effect on the size of the polypeptides observed. The results of using antiserum to the TEV NIb protein to immunoprecipitate the products of a translation carried out in the presence of amino acid analogues is shown in Fig.5.8 lane 7. Although lane 7 appears to have higher molecular weight products than lane 4 in Fig.5.8, which is a standard translation immunoprecipitated with TEV NIb antiserum, the original fluorograph does not show any difference between the two lanes.

The inclusion of antiserum to the TEV 49 kDa putative protease could have been expected to prevent processing of the polyprotein, but no effect on the size of the polypeptides was observed (Fig.5.7 Lane 3). Similar results were observed even when both the antiserum to the TEV 49 kDa protein and the amino acid analogues were included in the translation mix (data not presented). While the inclusion of antiserum to the TEV 49 kDa protein, the amino acid analogues and the amino acid analogues plus the TEV 49 kDa antiserum also did not alter the size distribution of the polypeptides, it did alter the abundance of the proteins. In all cases the bands appeared to be less intense and by inference less abundant. This was not due to alteration to the KM concentration, which would have accounted for the smaller quantity of accumulating proteins, because care was taken to ensure the concentration of KM remained optimal. Pre-immune rabbit serum added to the translation mix and included as a control (Fig.5.7 lane 4) appeared to inhibit the accumulation of bands with molecular weights of approximately 68-86 kDa. This included the 86 kDa protein which constitutes the major band in translations. The lower molecular weight bands, 68 kDa and below, all appear to be present. No explanation for this observation is apparent.

The effect of additional DTT on proteolytic processing is shown to be minimal (Fig.5.4 lane 2). Although DTT does not affect the size distribution of the proteins produced by the rabbit reticulocyte lysate, it would appear to affect the incorporation of the label. The intensity of the signal in lane 2 (Fig.5.4) is not as strong as in lane 4 of the same figure. Both lanes 2 and 4 have optimal KM concentrations.

Time Course Experiments

Standard 10 μl translation mixtures were incubated at 37°C for 2, 6, 10, 20, 30, and 50 minutes in an attempt to observe the order of accumulation of the proteins. Fluorography of the translation products revealed that small ^{35}S labelled polypeptides up to approximately 50 kDa accumulated during the first 10 minutes. After this time, polypeptides of higher molecular weights (98 kDa) began to accumulate. These species included at least 8 of the 11 major proteins observed in the standard reaction (data not presented).

To get a clearer indication of the accumulation of a specific protein (NIb) and its related precursors a standard 16 ul translation mixture was incubated for 5, 10, or 15 minutes. After incubation the products were immunoprecipitated with TEV NIb antiserum. The results are shown in Fig.5.8 lanes 8, 9, and 10. Only very small amounts of low molecular weight proteins accumulated after 5 minutes. After 10 minutes of translation, some products with molecular weights of approximately 30-50 kDa had accumulated. These did not include the putative NIb. After 15 minutes, polypeptides slightly smaller than the normal size distribution but up to approximately 110 kDa and including the NIb protein, were observed.

5.3.3 Immunoprecipitation of Proteins from MDL Translations

The immunoprecipitation experiments were aimed at identifying PSbMV gene products synthesised in *in vitro* translations. The results are presented in Fig.5.8 lanes 2, 3, 4, 5, and 6. Fig.5.8 is a composite of two different gels run to the same length. The bands from the different gels cannot therefore be considered to co-electrophorese.

The result of immunoprecipitation with PSbMV CP antiserum is shown in Fig.5.8 lane 2. The PSbMV CP antiserum immunoprecipitated a number of polypeptides with sizes larger than 33 kDa. Their molecular weights are summarised in Table 5.3. Fig.5.8 lane 2 shows that the most abundant polypeptide has a molecular weight of approximately 43 kDa. This band appeared in every experiment where PSbMV CP antiserum was used to immunoprecipitate the translation products. It did not, however, always appear to be as abundant as is seen in lane 2. The presence of this band is inexplicable as it does not have a molecular weight that could be associated with the CP. The band arrowed as representing the CP was chosen because it is present only in this lane, and its size, 33 kDa, corresponds to the size established by Western blots, SDS-PAGE and nucleotide sequence analysis. A precursor cross-reacting with the PSbMV

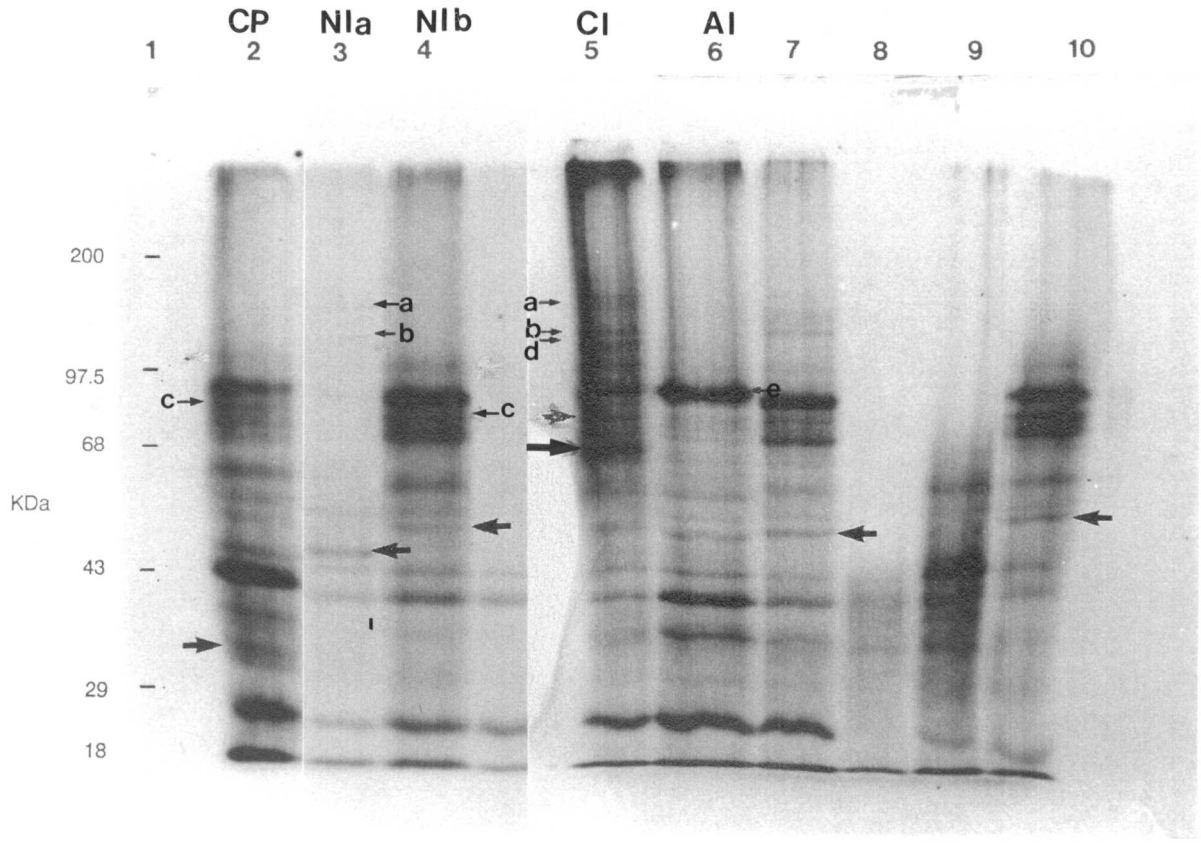


Fig.5.8. Analysis of the *in vitro* translation products of PSbMV-RNA using immunoprecipitation by specific antisera. **Lane 1.** Markers (see Fig.5.5). **Lane 2.** Products immunoprecipitated with antiserum to the PSbMV CP. **Lane 3.** Products immunoprecipitated with antiserum to the TEV NIa protein. **Lane 4.** Products immunoprecipitated with antiserum to the TEV NIb protein. **Lane 5.** Products immunoprecipitated with antiserum to the PSbMV CI protein. **Lane 6.** Products immunoprecipitated with antiserum to the WMMV AI protein. **Lane 7.** Products of a translation carried out in the presence of amino acid analogues and immunoprecipitated with antiserum to the TEV NIb protein. **Lanes 8-10.** Products immunoprecipitated with TEV NIb after 5, 10, and 15 minutes of translation. The small arrowed bands represent serologically cross-reacting proteins and the large arrowed unique bands to specific antisera.

Table 5.2

Gene combinations	Predicted MW	EVIDENCE FROM		
		Westerns	Translations	Immuno-precipitations
42k + CI + NIa + NIb	238		+	
32k + AI + 42k + CI	198			
CI + NIa + NIb + CP	196		+	
42k + CI + NIa	157		+	+
32k + AI + 42k	128			
CI + NIa	115		+	+
42k + CI	112		+	+
AI + 42k	96			
32k + AI	86		+	+
NIb + CP	81		+	+
CI	70	+	+	+
AI	54			
NIb	48	+	+	+
NIa	45		+	+
42k	42			
CP	33	+	+	+
32k	32			

Table 5.2. The origin of information used to construct the gene map proposed in Fig.5.6. Possible gene combinations and the predicted molecular weights of their products are included.

Table 5.3. The approximate molecular weights of proteins (seen in Fig. 5.8) immunoprecipitated with different antisera.

		Antisera		
PSbMV CP	TEV NIa	TEV NIb	PSbMV CI	WMMV AI
	157		**157**	
	115		**115**	
			112	
94		94	94	
				86
83	83	83	83	
81		**81**		
			70	
69	69	69	69	69
68			68	
			62	62
58	58	58	58	58
53	53	53	53	53
51	51		51	51
		48		
	45			
43	43	43	43	43
41	41	41	41	41
37		37	37	37
33				
31		31		
22	22	22	22	22

CP and TEV NIb antisera is indicated by the small arrow (c). This precursor has a molecular weight of 81 kDa.

Immunoprecipitation of *in vitro* translation products with TEV NIa antiserum also produced a number of bands (Fig.5.8 lane 3). The molecular weights of these are summarised in Table 5.3. In lane 3, the large arrowed band was chosen as the NIa protein because it is the most abundant and does not appear in other lanes. The molecular weight of this band (45 kDa) also corresponds to a band that was a candidate for the NIa protein in the Western blots. Two polypeptides cross-reacting with the TEV NIa and PSbMV CI antisera are arrowed (a and b). The estimated molecular weights of these two bands are 115 and 157 kDa. The bands are very faint in this lane even on the original fluorograph.

In Fig.5.8, lane 4 shows the result of immunoprecipitating products of an *in vitro* translation with TEV NIb antiserum. In lane 4, the band chosen as the NIb protein was not the most abundant protein. However, it had the molecular weight estimated by Western blot (48 kDa) and was not observed to be immunoprecipitated with antisera to the PSbMV CP, TEV NIa, PSbMV CI, and WMMV AI proteins. The precursor cross-reacting with the CP antiserum is arrowed (c) and has an estimate molecular weight of 81 kDa. The molecular weights of the numerous other polypeptides immunoprecipitated with the TEV NIb antiserum are summarised in Table 5.3.

The results of immunoprecipitation with PSbMV CI antiserum is seen in Fig.5.8 lane 5. The PSbMV CI antiserum immunoprecipitated a unique protein with a molecular weight of 70 kDa (large arrow). This was a major band and did not appear in the other lanes. The molecular weight of 70 kDa agrees with the molecular weight for this protein established by Western blots. The PSbMV CI antiserum immunoprecipitated a number of polypeptides (summarised in Table 5.3) including three high molecular weight precursors. Two cross-reacted with the TEV NIa antiserum (a and b) with estimated molecular weights of 115 and 157 kDa and the third (d) reacted with only the CI antiserum with a molecular weight of 112 kDa. The presence of this third possible precursor indicates there may be a protein with a molecular weight of approximately 42 kDa adjacent to the CI protein. The molecular weight of this third precursor (d) was calculated as the difference between the molecular weight of the observed precursor (112 kDa) and the known molecular weight of the CI protein (70 kDa). The difference is 42 kDa. A protein with a molecular weight of 42 kDa is postulated to be a gene product for potyviruses (Dougherty and Carrington 1988; Chang *et al.* 1988). However, a protein of this size has not been observed directly in infected plants and no antiserum to this protein is available.

The results of immunoprecipitating the products of *in vitro* translations with WMMV AI antiserum is seen in Fig.5.8, lane 6. While Western blots failed to give a clear indication of the size of the PSbMV AI protein so to did the immunoprecipitation experiments. As a consequence the size of the AI protein used in the analysis of data relies primarily upon published estimates for the molecular weights of other potyviral AIs. A protein immunoprecipitated only with the WMMV AI

antiserum is seen in lane 6. This arrowed band (e) has a molecular weight of approximately 86 kDa. The 86 kDa band is observed to have a slightly slower electrophoretic mobility than the major band appearing in lane 7. These two bands may represent the doublet observed in the translations. Because the smaller band appears in all the lanes except lane 6, its composition remains obscure. The presence of the 86 kDa precursor is supported by its appearance as the major band in translations. The 86 kDa protein is immunoprecipitated only with WMMV AI antiserum, suggesting that at least part of the AI protein comprises this precursor. This suggests that a protein of approximately 32 kDa may be adjacent to the AI protein. The molecular weight of 32 kDa was calculated by the difference between the observed size of the precursor (86 kDa) and the estimated molecular weight of the AI protein (54 kDa). As for the 42 kDa discussed above, a protein with a molecular weight of 32 kDa has not been observed directly in infected plants, and no antiserum is available.

Table 5.3 summarises the molecular weights of proteins produced in MDL programmed with PSbMV RNA and immunoprecipitated with different antisera. Proteins with molecular weights of 69, 58, 53, 43, 41 and 22 kDa were immunoprecipitated by all of the antisera. Three proteins (molecular weights 83, 51 and 37 kDa) were immunoprecipitated by four of the five antisera tested, although in each case the antiserun that did not immunoprecipitate the particular protein was different. One protein with a molecular weight of 94 kDa cross-reacted with three antisera (PSbMV CP, TEV NIb and PSbMV CI). Proteins with molecular weights of approximately 157, 115 and 81 kDa (bold type) and 68, 62 and 31 kDa cross-reacted with 2 antisera. Again, the cross-reacting antisera were not always the same pair. Proteins immunoprecipitated by only a single antiserum had molecular weights of 112, 86, 70, 48, 45 and 33 kDa (bold type). Four of these (70, 48, 45 and 33 kDa) probably represent mature proteins. The 86 and 112 kDa are probably precursors.

The molecular weights written in bold type in Table 5.3 represent polyproteins that are similar in size to that predicted by the sum of the molecular weights of the virus-specific proteins. The other cross-reacting proteins did not correspond to predicted sizes and were thus unable to be identified.

5.4 DISCUSSION

The first step towards determining the presence or absence of a sgRNA for the coat protein gene of PSbMV involved isolating viral RNA. There are advantages in isolating polysomes as an intermediate step towards extracting viral RNA. First, infected leaf tissue contains large quantities of host RNA relative to viral RNA (Otal and Hari 1983). Otal and Hari suggested that the RNA extracted from the polysome fraction could be expected to contain a high proportion of virus-related RNA. This prediction is supported by the observations (discussed in section 5.1) that some RNAs have specific characters which enable them to bind preferentially to the ribosomes

(Shapiro *et al.* 1988). Moreover, isolation of RNA associated with the ribosomes would select for the RNA that was actively being translated. An additional advantage in isolating RNA from polysomes is that polysomal RNA is less likely to be degraded since it is being actively translated. However, much of the viral RNA is likely to be sequestered in virions and is not available for translation.

The Northern blots indicated that a band corresponding to full length RNA was present in the RNA isolated from the infected polyribosomes. Both Vance and Beachy (1984a) and Dougherty (1983) observed similar results for SMV and TEV respectively. Their observations contradict the earlier report by Otal and Hari (1983), who postulated the presence of seven sgRNAs for TEV. My results do not support the latter proposal: no bands were present within the molecular weight region predicted for a coat protein sgRNA. Vance and Beachy (1984a) and Dougherty (1983) concluded that the sgRNAs observed by Otal and Hari (1983) were due to the degradation of genomic length RNA. My observation that the only PSbMV RNA of discrete length is associated with the polysomes is consistent with this conclusion. The degradation of the genomic RNA observed in the Northern blots was also reported by Dougherty (1983). He offered no explanation for the degradation of the TEV RNA. It may be noted, however, that some degradation of the RNA always accompanies the isolation of full length PSbMV RNA (see Chapter 2.4 for discussion).

All the available evidence indicates that PSbMV, like other potyviruses (for example TEV, TVMV, and SMV), is translated as a polyprotein which is processed to form a number of mature products. The standard rabbit reticulocyte lysate mixture indicated the presence of 7 major proteins with molecular weights larger than any mature viral protein. The largest of the 7 proteins has a molecular weight of approximately 238 kDa. This is considerably larger than the biggest mature protein, which is the cylindrical inclusion protein (70 kDa). These larger proteins suggest that unprocessed precursors composed of several proteins linked in sequence may be present. This conclusion was also reached by Vance and Beachy (1984b) for SMV and by Yeh and Gonsalves (1985) for PRV. Nucleotide sequence analyses of clone pPSb70 do not show an inframe AUG codon between the coat protein and the adjacent genes (Appendix A). In addition, the sequence data from pPSb70 indicate a cleavage site cassette (which is the amino acid sequence at which the protease cleaves). Hence the sequence data also strengthen the polyprotein hypothesis, at least for these two genes.

A gene map for PSbMV is proposed in Fig.5.6. This was constructed using a compilation of data from MDL-translations, immunoprecipitation of MDL translated products and Western blots. The main evidence has come from immunoprecipitation data. Here, three cross-reacting precursors were observed, as well as two bands corresponding to precursors that reacted with only one antiserum. Unique bands for CI, CP, NIa, and NIb were also observed. The presence of the unique bands confirms the molecular weights for the CP, NIb, and CI proteins, previously

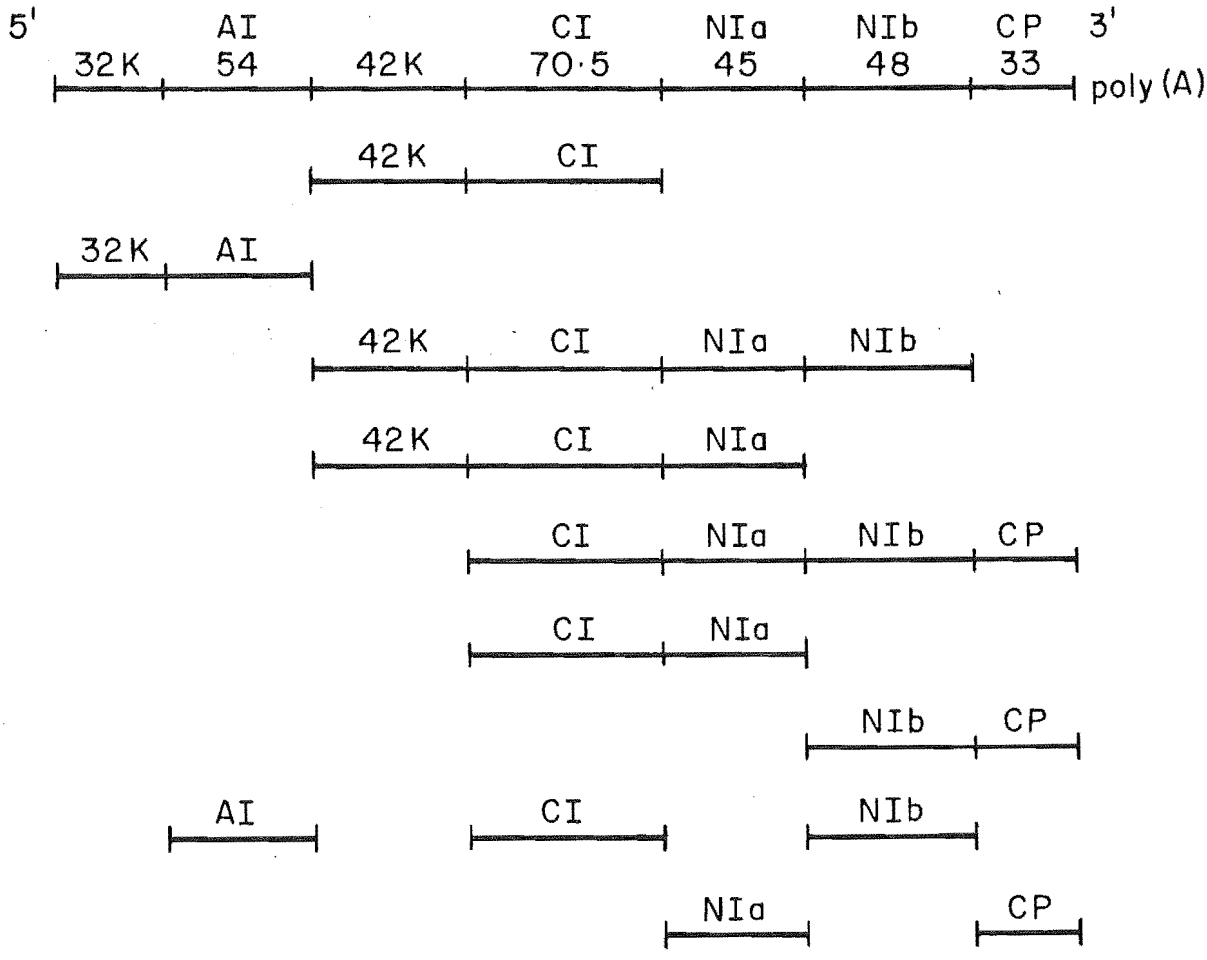


Fig.5.6. The proposed gene map for PSbMV. The molecular weights (x 10^3) of the putative cleavage products are shown. The origin of the supporting information is given in Table 5.2. The presence of the poly[A] tail is supported by sequence data and the dependence on oligo-(dT) primer for the production of first strand cDNA. This figure is not drawn to scale.

established by Western blots (see Chapter 4) and it establishes their viral origins. The molecular weight of the NIa protein was established by immunoprecipitation. The AI protein was not observed as a unique band. It has been suggested by other workers that the AI protein is not processed out of the polyprotein in rabbit reticulocyte lysate (Dougherty and Hiebert 1980c; R.E. Rhoads pers. comm.). Reasons for this are suggested later in this discussion. Some of the proteins (32 and 42 kDa) suggested to be encoded at the 5' end of the molecule were not directly observed in this study. There is, however, evidence of their size and map location in the literature (see Dougherty and Carrington 1988 for discussion). This information was used to help interpret data from the 5' end. The proposed map for PSbMV is supported by the interpretations of other workers. A similar genomic organisation has been postulated for BYMV (Chang *et al.* 1988), TVMV (Hellmann *et al.* 1986) and TEV (Allison *et al.* 1985).

A small genome-linked protein, the VPg, has been found in other potyviruses. The VPg has been shown to be covalently attached to the RNA genomes of TEV (Hari 1981) and TVMV (Shahabuddin *et al.* 1988). The size of the VPg is the subject of debate. The VPg of TEV appears to be 6 kDa in size (Hari 1981), while that of TVMV has an estimated molecular weight of 24 kDa (Siaw *et al.* 1985). The VPg has been mapped between the CI and NIa genes (see Dougherty and Carrington 1988). No antiserum to the VPg was available for my study so the presence of this protein for PSbMV remains unanswered.

All of the antisera used in the immunoprecipitation of MDL products immunoprecipitated a large number of polypeptides (Table 5.3). Among these, a number of polypeptides are observed that are similar in size to those predicted by the sum of the molecular weights of virus specific proteins (summarised in Table 5.2). Extra bands present in the translations may represent translation mistakes, degraded template, and/or degradation by non-specific proteolysis.

Some partially processed polyproteins cross-reacted with two antisera in the immunoprecipitation experiments. In my study, a precursor with a molecular weight of 81 kDa cross-reacted with the PSbMV CP and TEV NIb antisera. A similar precursor with a molecular weight of 82 kDa was observed by Chang *et al.* (1988) for BYMV who suggested that it represented the NIb+CP precursor.

Two proteins cross-reacted with the TEV NIa and PSbMV CI antisera. The size of one of the proteins (115 kDa) agrees with the sum of the molecular weights for the CI and NIa proteins. Chang *et al.* (1988) determined that this precursor of BYMV has a molecular weight of 120 kDa and suggested this represented the CI+NIb precursor. The slight difference in values may be due to the difference in the molecular weights of the mature components. The second protein cross-reacting with the TEV NIa and PSbMV CI antisera had a molecular weight of 157 kDa. The molecular weight corresponds to the predicted size of the 42K+CI+NIa precursor using the sum of the molecular weights for the NIa+CI (determined in this study) and the 42K suggested by the literature and also tentatively calculated here. Chang *et al.* (1988) observed a similar precursor.

The information from the proposed 5' end of the genome was more difficult to interpret because the molecular weights of the proteins flanking the AI protein are speculative. Although these mature products from the 5' end of the genome have not been observed in infected plants, both Chang *et al.* (1988) and Dougherty and Carrington (1988) have tentatively proposed molecular weights for them. The molecular weights proposed by Chang *et al.* (1988) for the two proteins flanking the AI were suggested to be 32 kDa and 42 kDa. In my study, two high molecular weight proteins were immunoprecipitated only by a single antiserum. The first of the two high molecular weight proteins was immunoprecipitated by PSbMV CI antiserum and had a molecular weight of 112 kDa. This precursor may correspond to the 42K+CI precursor as suggested by Dougherty and Carrington (1988) and Chang *et al.* (1988). Chang *et al.* detected a precursor comparable to the 112 kDa protein with a molecular weight of 115 kDa. A second protein that reacted with only one antiserum (WMMV AI) had an estimated molecular weight of 86 kDa. This precursor may represent the 32K+AI precursor. Again, the literature supports the presence of a protein with a molecular weight of 32K (Dougherty and Carrington 1988; Chang *et al.* 1988).

The faintness of the bands observed for most of the high molecular weight precursors is best attributed to the factors suggested by Vance and Beachy (1984b) and Carrington and Dougherty (1988). They proposed that the processing of the polyprotein is extremely rapid and that at any one time the quantity of high molecular weight precursors is likely to be extremely low. Their proposal is supported by the results presented in this chapter and in Chapter 4, where high molecular weight polyproteins were not detected in Western blots. In the light of this observation, the ability to inhibit the processing of the polyprotein would have been invaluable. A possible alternative would have been to significantly increase the incubation time of the MDL. A longer time may have given more high molecular weight precursors, especially the products encoded at the 3' end of the genome.

The standard translation mixture produced more than 11 major proteins from PSbMV RNA. Other workers have observed similar results. For example, Vance and Beachy (1984b) found that SMV produced 10-12 major proteins, while Shields and Wilson (1987) observed more than 8 major proteins for turnip mosaic virus (TuMV) and Dougherty and Hiebert (1980a) found 5 major proteins for both PeMV and TEV. The major cell-free translation product of PSbMV-RNA is an 86 kDa protein. Dougherty and Carrington (1988) observed that TEV and TVMV produced major products of 87 and 75 kDa repectively. The presence of this precursor as a major band has several interesting implications. First, it is suggested that the 32K+AI proteins, which may comprise this precursor, are encoded at the 5' end of the genome (Dougherty and Hiebert 1980c; Hellmann *et al.* 1980). This abundance implies that the products of this part of the genome, which is translated first, may accumulate in greater than equimolar amounts because the ribosomes "fall off" the RNA during translation. Thus the downstream genes, which do not have a ribosome

binding site or a start codon, would not be translated and their products would be present in much lower concentrations. It is striking that in picornaviruses, regarded by most workers as being similar to potyviruses (Argos *et al.* 1984), this 5' position is occupied by the gene for the coat protein (Kitamura *et al.* 1981). This strategy seems more reasonable because the virus presumably requires many more coat protein units than enzymes such as the replicase (NIb). Second, R.E. Rhoads (pers. comm.) has observed that the cleavage of the products encoded at the 5' end of the TVMV genome is delayed in *in vitro* translations for some reason. He also suggested that these unprocessed molecules may have an additional role in replication. In an example from another plant virus group, Dorssers *et al.* (1984) observed that a 110 kDa precursor from cowpea mosaic virus is responsible for RNA chain elongation. The 110 kDa protein is encoded at the 3' end of the B RNA of cowpea mosaic virus. The regulation of both viral replication and processing of the potyviral polyprotein is not yet understood.

The time course experiments for both the translations and the immunoprecipitation of the translated products indicate that small proteins appear first and larger precursors are seen only later. These results are supported by other workers. Vance and Beachy (1984b), Dougherty and Hiebert (1980a), and Hellmann *et al.* (1980) all reported that the smallest translation products appear early in the reaction. The absence of early accumulation of higher molecular weight precursor molecules may be caused by the rapid processing of the polyprotein. The processing may even occur while the polyprotein is still associated with the polyribosomes. Alternatively, the MDL may not have had sufficient time to produce high molecular weight molecules. It is noted that in the time course experiments the NIb protein did not appear until after 15 minutes incubation. However, the NIb protein has been mapped adjacent to the CP which is encoded at the 3' end of the molecule, so the precursor cleaved to produce the NIb protein presumably must have been synthesised.

Previous workers have attempted to incorporate amino acid analogues in order to block or slow the proteolytic cleavage of potyviral polyproteins. The technique has met with mixed success. The accumulation of high molecular weight precursors of SMV, which was observed by Vance and Beachy (1984b) was supposedly caused by the analogues altering the cleavage cassette site for the protease. Not all workers have observed this phenomenon, however. Shields and Wilson (1987) working on TuMV, Yeh and Gonsalves (1985) using PRV, and this study all failed to observe any inhibition of proteolytic processing when amino acid analogues were included in the rabbit reticulocyte lysate translation mix. Yeh and Gonsalves (1985) went so far as to suggest that primary cleavage was mediated by a lysate component and that consequently the inclusion of analogues would have no effect on the processing. It is now recognised that this is incorrect (Hellmann *et al.* 1988; Carrington and Dougherty 1987b). An explanation for the failure of amino acid analogues to inhibit processing of PSbMV polyprotein can be suggested. The amino acid analogues for arginine (R), lysine (K) and phenylalanine (F) were used in this study.

The cleavage site cassette for PSbMV between the CP and NIb protein is V R L Q/ A (Appendix A. The slash indicates the probable site of cleavage). It is apparent that only one site of this cassette will have an amino acid analogue substitute. This may not be enough to affect the recognition and subsequent cleavage of the PSbMV polyprotein. Moreover, the presence of endogenous amino acids in the lysate mixture, even at low concentrations, enables their incorporation into the polyprotein at the cleavage cassette site. Yeh and Gonsalves (1985) attempted to eliminate the endogenous amino acids from their system, but this made no difference to the processing of the polyprotein. Processing of the polyprotein could also be eliminated if the active site of the protease was altered. A change could occur if the amino acid analogues were incorporated at the active site of the enzyme.

Several workers have observed that DTT was necessary for the processing of the potyvirus polyprotein (Yeh and Gonsalves 1985; Chang et al. 1988). Shields and Wilson (1987), and this study, have found no differences in the molecular weights of accumulating proteins in the rabbit reticulocyte lysate system in the absence of DTT. It is important to note that in both these studies, as well as those conducted by Yeh and Gonsalves, a small amount of reducing agent from the [^{35}S] methionine stock was present. In addition, approximately 0.4 mM DTT was present from the amino acid stock used in this study and that conducted by Shields and Wilson. Thus, a small amount of reducing agent was present even before more DTT was added. E. Hiebert (pers. comm.) has suggested that the PSbMV protease may not be as sensitive to the absence of reducing agent as the BYMV protease in the study conducted by Chang *et al.* (1988).

Translation in the presence of the TEV 49 kDa antiserum, which is the putative protease for the 3' end proteins (Hellmann *et al.* 1988; Domier *et al.* 1987; Carrington and Dougherty 1987b) did not inhibit cleavage of the PSbMV polyprotein. E. Hiebert (pers. comm.) has suggested that the antiserum to the 49 kDa (NIa) of TEV may not interact with the PSbMV protease at the active site for protease activity. If this is so, the protease activity would not be nullified. Western blots indicate that TEV NIa antiserum does not interact well with the PSbMV proteins (see Chapter 4). The sequence homology between the NIas of two other potyviruses (TEV and TVMV) is low, 48.8%. Only the carboxyl-terminal halves of TEV and TVMV proteases exhibit sequence similarity (Domier *et al.* 1987). However, the cleavage cassette envisaged by Carrington and Dougherty (1988b) and Hellmann *et al.* (1988) for NIa suggests that the protease should exhibit significant conformity and hence sequence homology in the active site region. The cleavage site cassette between the CP and NIb protein of PSbMV is suggested to be V R L Q/ A (Appendix A). Both TEV and TVMV exhibit different cleavage site cassettes, although the cleavage site cassette for TVMV is similar to that of PSbMV. The TEV cassette is E X X Y X Q/ S or G (Carrington and Dougherty 1988b), while the regions flanking the cleavage sites of TVMV are characterised by V R F Q/ S or V R T Q/ S (Hellmann *et al.* 1988). Depending on how the antiserum was prepared, such changes may be enough to prevent interaction of the TEV 49 kDa antiserum from TEV with

the Nla protein from PSbMV. This is particularly likely because the rest of the molecule has been shown to have low sequence similarities with the two other potyviruses discussed. It is significant that Chang *et al.* (1988), who observed the inhibition of polyprotein processing for BYMV by the 49 kDa antiserum, used antiserum made from the Nla protein of BYMV. Obviously most of the problems outlined above would be eliminated with homologous sera. The present study used antiserum made to the Nla protein of TEV in experiments with PSbMV proteins. An alternative suggestion, that different potyviruses have nonidentical active sites, would also account for the observations discussed above.

In conclusion, a gene map has been proposed for PSbMV using data from a number of different sources. The translation strategy of PSbMV appears to be the production of a polyprotein that is cleaved to produce a number of mature products whose molecular weights differ from comparable proteins in other potyviruses.

CHAPTER 6

ELECTROPORATION OF PROTOPLASTS OF *Nicotiana* SPP. WITH PSBMV-RNA AND WHOLE VIRUS

6.1 INTRODUCTION

The introduction of infectious RNA into plant cells provides a mechanism for studying plant virus infection at the cellular level. A considerable amount of information concerning the organisation of the RNA genomes of some potyviruses is now available. Most of this information has been gained from *in vitro* translations in either wheat germ (for example Koziel *et al.* 1980) or rabbit reticulocyte lysate (see Chapter 5.0). Relatively little is known about potyvirus expression in infected plants cells, however.

The main problems associated with any whole plant system are the low efficiency of initial infection and the property that virus multiplication is not synchronous (Sakai *et al.* 1977). These two problems are largely overcome by the use of protoplast systems (Takebe 1975). Since the first reported infection of *Nicotiana tabacum* protoplasts with TMV-RNA (Aoki and Takebe 1969), various procedures have been proposed for introducing viral RNAs into plant protoplasts. Cationic polymers such as poly-L-ornithine have been used to infect tobacco protoplasts with TMV-RNA (Aoki and Takebe 1969) and cowpea chlorotic mottle virus (CCMV) and CCMV-RNA (Motoyoshi *et al.* 1973). Polymers such as PEG have also been used to aid the cellular uptake of RNA and whole virus; for example, Maule *et al.* (1980) used PEG to introduce cucumber mosaic virus and viral RNA into cucumber protoplasts. Alternatively, viral RNA can be encapsulated in liposomes which are introduced into protoplasts with the aid of PEG or polyvinyl alcohol (Nagata *et al.* 1981; Fraley *et al.* 1982) The first electroporation experiments showed that electric impulses significantly increased the uptake of DNA by mouse L cells (Neumann *et al.* 1982). Since then, plant virologists have also used this method. Watts *et al.* (1987) suggested that electroporation enhanced translation by an order of magnitude compared to the more conventional techniques.

A variety of techniques are used to detect viral proteins in protoplasts. Many workers use fluorochrome-labelled antibodies (Okada *et al.* 1986; Aoki and Takebe 1969; and Maule 1983). A second related method uses immunogold labelling, as was done by Steffenson *et al.* (1987) to detect geminiviruses in legume protoplasts, and Roenhorst *et al.* (1988) to detect CCMV in cowpea protoplasts. Many workers also use gel electrophoresis and autoradiography to observe radiolabelled proteins produced in virus-infected protoplasts. The inclusion of Actinomycin D during the incubation period after electroporation has eased the task of deciding which bands

are host-labelled proteins and which are virus-encoded proteins. The inclusion of Actinomycin D should not affect the transcription of RNA viruses because it is an inhibitor of DNA-dependent RNA-polymerase. Some workers have reported that Actinomycin D interferes with the transcription of picornaviruses (Cooper 1966; Grado *et al.* 1965; and Schaffer and Gordon 1966), but this problem does not appear to be a general characteristic. Using Actinomycin D, Rottier *et al.*(1979) were able to detect the presence of 6 virus-specific proteins in CPMV-infected protoplasts. Watanabe *et al.* (1984) detected TMV-specific proteins in tobacco. The following detection techniques are used in this study- immunogold labelling, fluorescent labelled antibodies, fluorography and Western blots.

The experiments presented in this chapter have two aims. The first was to show that the proteins produced in the MDL were a faithful representation of those found in plant cells. The second aim was to determine whether electroporation of whole virus or viral RNA could be used to study the replication of PSbMV in non-host (tobacco) protoplasts. Questions that can be addressed include: 1) the ability of the virus to uncoat in tobacco protoplasts; 2) the ability of the RNA to be translated in tobacco protoplasts; and 3) the ability of the virus to replicate in tobacco protoplasts. In this study, various techniques (referred to above) are used to detect virus products in infected protoplasts. Inoculation of the local lesion host for PSbMV, *Chenopodium amaranticolor*, was also used as an additional method for detecting the production of virions in protoplasts.

6.2 MATERIAL AND METHODS

6.2.1 *Nicotiana clevelandii* and *N. tabacum* as Hosts for PSbMV

Inoculation of Nicotiana Plants

Duplicate plants of *N.clevelandii* and *N. tabacum* were inoculated with infected pea material as follows: infected pea leaf tissue (1 g) was ground in a mortar and pestle with 1.5 ml of Yarwood's buffer (0.5% K_2HPO_4, 0.5% bentonite, Yarwood 1968). Two leaves of each tobacco plant were lightly dusted with 400 mesh carborundum powder and the "infected paste" was rubbed on. Additional plants of each tobacco species were mock-inoculated with dH_2O. The leaves were immediately rinsed with dH_2O and the plants grown in an insect-free growth room at 16°C with a 16 hour light period.

6.2.2 Search for Symptoms

Visual Inspection and Electron Microscope Examination

Inspection for visible symptoms began two weeks after inoculation and continued for 6 weeks. Grids for immunotrapping were coated with the coat protein antiserum to PSbMV. The formvar-coated grids were prepared by floating them in a drop of CP antiserum at a 1:500 dilution in PBS (4 mM KH_2PO_4, 16 mM Na_2HPO_4 and 115 mM NaCl pH 7.3) for 30 minutes at room temperature. The coated grids were then washed 3 times for 5 minutes each time in PBS. Samples were taken from inoculated plants at one week intervals as outlined in section 2.2.2. The plant supernatant was left on the grids for 5 minutes before draining and staining with PTA (see section 2.2.1).The grids were not allowed to dry before the application of the negative stain. Grids were examined by transmission electron microscopy.

ELISA Test

ELISA tests were conducted on the tobacco plants one month after inoculation to determine whether the plants were infected with PSbMV. The standard test was conducted by J. Fletcher, Plant Diseases Division, DSIR, Lincoln, using antiserum from the same source used for the Western blots and immunoprecipitation studies (Mink).

RNA Dot Blots

This procedure follows the method outlined in the Gene Screen Plus manufacturer's manual. All procedures used RNase-free materials (see Chapter 2.2.1). After one month, 0.05 g of plant material from the inoculated and mock-inoculated tobacco plants was ground in 0.5 ml of extraction buffer (50% deionised formamide and 6% formaldehyde). The sample was centrifuged at 5,000 g for 5 minutes. 200 μl of the supernatant was left undiluted while a 20 μl aliquot was diluted to 200 μl in the extraction buffer. Controls containing 10 pg of purified viral RNA and 12 pg of unlabelled probe were included. All samples were heated to 50°C for one hour to denature the RNA. The dot blot apparatus was assembled according to the manufacturer's instructions (Biorad). The denatured RNA was added to the slots where it was incubated for 30 minutes and then sucked through. The rest of the procedure follows that outlined in Chapter 5.2.4.

Electroporation of Protoplasts of *Nicotiana* spp. with PSbMV RNA

6.2.3 Isolation of Protoplasts

Protoplasts were isolated by a modification of the method of Chin and Scott (1979). Tobacco plants were grown under reduced light to produce "soft" plants suitable for electroporation. 0.1 g

of tobacco leaf (either *N.clevelandii* or *N.tabacum*) was rinsed in 0.6 M mannitol and 10 mM $CaCl_2$ adjusted to pH 6.8 with HCl. The mid-rib of the leaf was excised and the lamina cut into 1 mm slices with a new razor blade. The slices were immediately immersed in prewarmed incubation medium (27°C) containing 2% cellulase ("Onozuka" RS), 0.5% macerozyme R-10 (both from Yakult Honsha Co Ltd) and 1.0% hemicellulase (Sigma) in 0.6 M sorbitol and 10 mM $CaCl_2$ and adjusted to pH 6.8 with HCl. The sample was vacuum infiltrated for 5 minutes. To produce protoplasts, the sample was incubated for approximately 1.5 hours at 27°C with shaking at approximately 80 strokes a minute. The protoplasts were decanted through 4 layers of miracloth and pelleted at 50 g in a bench top centrifuge for two minutes. The incubation medium was aspirated off and the protoplasts were washed 3 times with ice cold 0.5 M mannitol. The protoplasts were counted using a haemocytometer, and their concentration was adjusted to approximately $1x10^6$ protoplasts per ml.

6.2.4 Electroporation Reaction

10 μg of PSbMV RNA was added to one ml of protoplast suspension, and immediately electroporated at 200 V and 330 μF capacitance (BRL Cell-porator) on ice. The protoplasts were left on ice for 30 minutes, then collected by centrifugation at 50 g for two minutes. The cells were washed with one ml ice cold 0.5 M mannitol and 10 mM $CaCl_2$ adjusted to pH 6.8 with HCl to remove residual RNA. The protoplasts were again collected by centrifugation and resuspended in 1 ml 0.5 M mannitol, 2% sucrose, 30 μg Actinomycin D and 10 μCi [^{35}S] methionine. The suspension was incubated in the dark for 18 hours at 25°C. Controls without Actinomycin D and mock-inoculated protoplasts (ie subjected to electroporation in the absence of viral RNA) were included. After incubation, the protoplasts were collected by centrifugation, washed once with 0.6 M mannitol and respun.

6.2.5 Analysis of Proteins Produced in Protoplasts

Electrophoresis

The pellets were resuspended in 2 x sample buffer (Laemmli 1970) and heated at 100°C for 3 minutes to lyse the cells and denature the proteins. SDS-PAGE gels were prepared as described in section 5.2.11. The entire sample was loaded onto the gel and the radiolabelled proteins were detected by fluorography.

Western blot analyses

The pellets were prepared for electrophoresis as described above. The entire sample was loaded onto a 10% polyacrylamide gel and electrophoresed as described in section 4.2.4. The proteins

were transferred to NCM and Western blots were conducted using the antiserum to the coat protein of PSbMV as described in section 4.2.5.

Immunofluoresence

The procedure for immunofluorescent staining is described in Chapter 3. The antisera to the CP or CI (1:500 dilution) were used as the 1° antisera, and donkey anti-rabbit conjugated to fluorescein isothiocyanate at a dilution of 1:20 was the 2° antiserum. A "blind" experiment was conducted as described in section 3.2.2. Protoplasts were examined with a Lietz epiflurescent microscope and photographed on Kodacolor 400 ASA.

6.2.6 Time Course Experiments

Protoplasts of *N.tabacum* and *N.clevelandii* were electroporated with PSbMV-RNA as described above, except that the [^{35}S] methionine was omitted. Protoplasts were incubated for 0, 6, 12, and 18 hours prior to inoculation of *Chenopodium amaranticolor*.

Times used for RNA dot blots (section 6.2.7) were 0, 4, 8, 12, 16, 20, and 24 hours. Mock-electroporated controls were included.

6.2.7 Detection of Virus Replication and Translation in Protoplasts

Inoculation of Chenopodium amaranticolor with incubated protoplasts

From each tube, 250 μl of mixed protoplast suspension were withdrawn at the time stated above (6.2.6). The protoplasts were pelleted by centrifugation at 50 g and washed 3 times with 0.6 M mannitol to remove any lysed cells and free RNA. The cells were resuspended in 100 μl 0.5 M PBS pH 7.0. This molarity of PBS lysed the cells and released the viral RNA and/or virus particles. Young, three-quarters expanded *C.amaranticolor* leaves of equal status were chosen for inoculation. Each leaf was dusted with 400 mesh carborundum powder, and the 100 μl samples were gently rubbed into the leaves. The leaves were immediately rinsed in dH_2O to remove the carborundum powder. Controls using mock-electroporated protoplasts (with dH_2O) and mock inoculation of leaves with dH_2O were included. Two positive controls, one of which was 200 μl of freshly prepared inoculum from infected peas (see section 6.2.1) and the other of 1 μg of purified PSbMV RNA in 0.5 M phoshate buffer pH 7.0, were included.

The plants were maintained in an insect-free growth room at 16°C and 16 hour light period for 4 weeks. Plants were examined daily for the appearance of local lesions.

RNA Dot Blots

140 μl of protoplast suspension was removed from the incubating tubes of *N.clevelandii* and *N.tabacum* at the times specified above. Each sample was centrifuged at 50 g in a microfuge to pellet the protoplasts. The cells were washed 3 times with 100 μl 0.6 M mannitol. The final washing solution was reserved for dot blot analysis to check the amount of RNA washed off the outside of the protoplasts. The pelleted protoplasts were disrupted by freeze/thawing once at -80°C. 50 μl of sample buffer (50% deionised formamide and 6% formaldehyde) was added to the pellet. 100 μl of sample buffer was added to the final washings. Controls of 10 pg of unlabelled probe (pPSb70) and 10 pg of purified virus RNA were treated similarly. The procedure for the remaining steps is the same as that described in section 6.2.2.

Examination with the Transmission Electron Microscope

Grids were dipped into the suspension of intact protoplasts, drained and negatively stained as described in section 2.2.1.

6.2.8 Immunoprecipitation of Viral Proteins

The protoplasts were electroporated as described above (section 6.2.4). After 18 hours of incubation, the protoplasts were pelleted by centrifugation, frozen briefly at -80°C to lyse the cells and resuspended in 60 μl 0.75 M NaCl/1% Triton X-100. The following were added to each sample: 8 μl of PSBMV coat protein antiserum, 60 μl 62.5 mg ml^{-1} protein A Sepharose in 25 mM Tris-HCl pH 7.5 and 3% bovine serum albumin (to reduce non-specific binding). The samples were then gently mixed overnight at 4°C. The immunoadsorbed proteins were recovered by centrifugation at 8,000 g for two minutes. The pellets were resuspended in 2 x Laemmli sample buffer, heated at 100°C for 3 minutes, and cooled on ice. The supernatant was collected following centrifugation and a further 10 μl 2 x sample buffer was added to the pellet. The sample was again centrifuged and the supernatants pooled. These were analysed on 10% polyacrylamide gels and the presence of radiolabelled proteins detected by fluorography.

6.2.9 Electroporation with Whole Virus

Protoplasts were prepared as described in section 6.2.3. The standard reaction was set up except that 40 μg ml^{-1} of whole virus from one of two sources was used as inoculum in place of the viral RNA. In addition, a third source of inoculum containing a minimum of 10 μg ml^{-1} of virus was used. The sources of inoculum were: a) whole virus from a previous virus purification stored at -80°C; b) partially purified virus; c) fresh inoculum from an infected pea plant. Partially purified virus (b) was prepared using a shortened version of Reddick and Barnett's (1983) procedure. Here, the procedure continued as outlined in section 2.2.3, but the final PEG pellet, instead of

being loaded onto a Cs_2SO_4, was resuspended in 2 mls of 20 mM Tris-HCl pH 7.0. The sample was dialysed overnight against two litres of 20 mM Tris-HCl pH 7.0 with two buffer changes. The concentration of the virus in both the stored wole virus and the partially purified sample were determined spectrophotometrically and the appropriate volume was added to the protoplasts for electroporation. Fresh inoculum (c) was prepared by homogenising 0.5 g infected plant material in 1 ml 0.5 M mannitol and centrifuging at 13,000 g in a microfuge for 10 minutes. The supernatant (1 ml) was used to resuspend the protoplasts after their isolation. The concentration of virus in the fresh material was determined by applying the estimated amount of coat protein in a volume of infected plant material using Western blots. Electroporation was at 200 V and 330 μF. The rest of the procedure follows section 6.2.4.

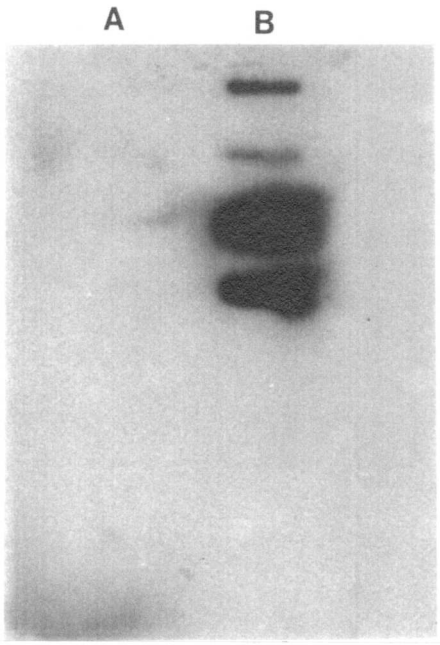
6.3 RESULTS

6.3.1 *N. clevelandii* and *N.tabacum* as Host Plants for PSbMV

Observations were made on the *Nicotiana* plants inoculated with PSbMV-RNA. No visible symptoms were apparent during the 6 weeks of observation. Examination of plant material from the upper (systemic) leaves on the immunotrapping grids using the electron microscope detected no virus particles. Likewise the ELISA test for PSbMV and the RNA dot blots (Fig.6.1) preformed on the upper leaves of inoculated plants were negative. The controls for the RNA dot blots of 10 pg of PSbMV RNA and 12 pg of probe both showed a positive reaction. The results indicate that the two species of tobacco were not systemically infected by PSbMV.

6.3.2 Electroporation of *Nicotiana* spp. Protoplasts with PSbMV RNA

Protoplasts of *Nicotiana* spp. were electroporated with PSbMV-RNA and incubated in the presence of [^{35}S] methionine. The resulting proteins were analysed by SDS-PAGE and the radiolabelled proteins were detected by fluorography. The fluorograph of ^{35}S labelled proteins (Fig.6.2) revealed 4 bands that were present in the protoplasts electroporated with PSbMV-RNA (Lane 3) but were absent in the mock-electroporated protoplasts (Lane 2). The estimated molecular weights for the proteins unique to the protoplasts electroporated with PSbMV-RNA (Lane 3) were 86, 70, 54 and 48 kDa. These correspond to the predicted molecular weights of a number of virus-encoded proteins. The proteins in the various bands may therefore represent the following virus-encoded proteins: CI (70 kDa), AI (54 kDa), NIb (48 kDa) and the 86 kDa protein. Bands which could represent the NIa or CP were not observed. Several bands from the rabbit reticulocyte lysate translations had similar electrophoretic mobilities (Lane 4) with the bands unique to the infected protoplasts. The major band, representing the 86 kDa (large arrow), is seen to have a similar mobility to the band arrowed (a) in the inoculated protoplasts. The band



A	B
1	9
2	10
3	11
4	12
5	
6	
7	
8	

Fig.6.1. RNA dot blot analysis of samples from *N.clevelandii* and *N. tabacum* plants 6 weeks after inoculation with PSbMV. Explanation of the grid is adjacent.

1 and **2**. *N. tabacum* inoculated with PSbMV. **3** and **4**. *N. tabacum* mock inoculated. **5** and **6**. *N. clevelandii* inoculated with PSbMV. **7** and **8**. *N. clevelandii* mock inoculated. **9** and **10**. 10 pg PSbMV-RNA. **11** and **12.** 12 pg of probe.

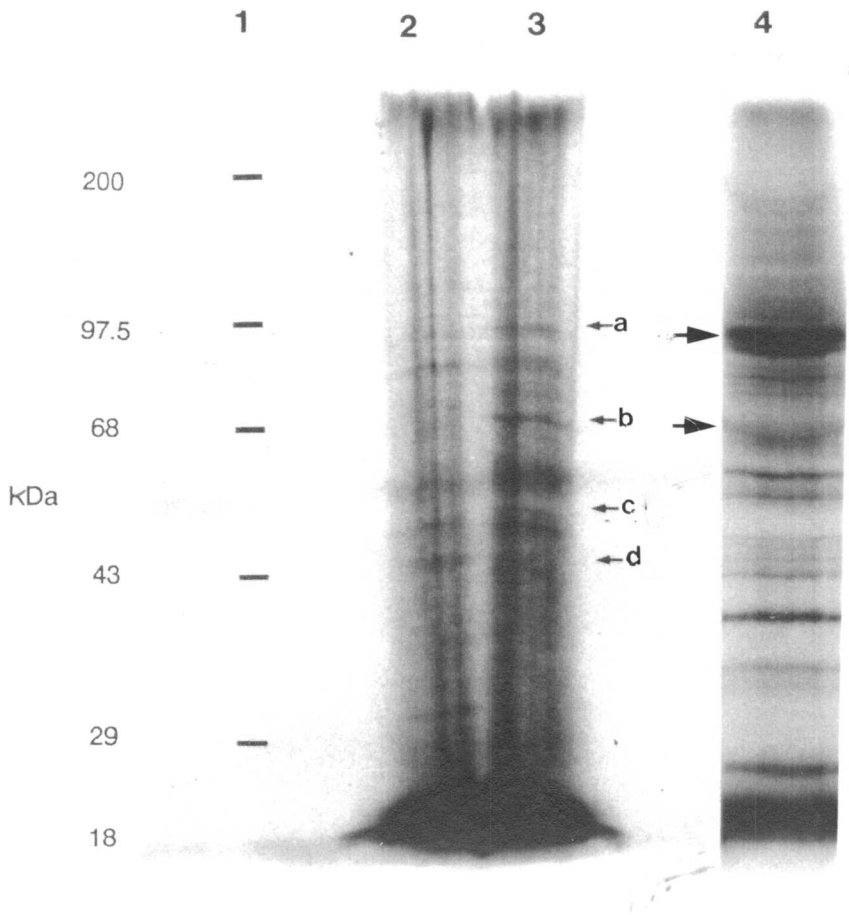


Fig.6.2. ^{35}S-labelled proteins produced after electroporation of N. *tabacum* protoplasts with PSbMV-RNA separated by SDS-PAGE and detected by fluorography. **Lane 1**.Pre-stained markers (BRL) (x 10^3 Da). Myosin (200), phosphorylase B (97.5), BSA (68), ovalbumin (43), carbonic anhydrase (29), and B-lactoglobulin (18). **Lane 2.** Proteins labelled in mock electroporated protoplasts. **Lane 3.** Proteins labelled in protoplasts electroporated with PSbMV-RNA. The bands unique to the protoplasts electroporated with PSbMV-RNA are arrowed (small arrow). **Lane 4.** S^{35}-labelled proteins produced in rabbit reticulocyte lysate programmed with PSbMV-RNA. The large arrows indicate bands observed in lane 4 that have similar electrophoretic mobilities to bands in lane 3 (a and b).

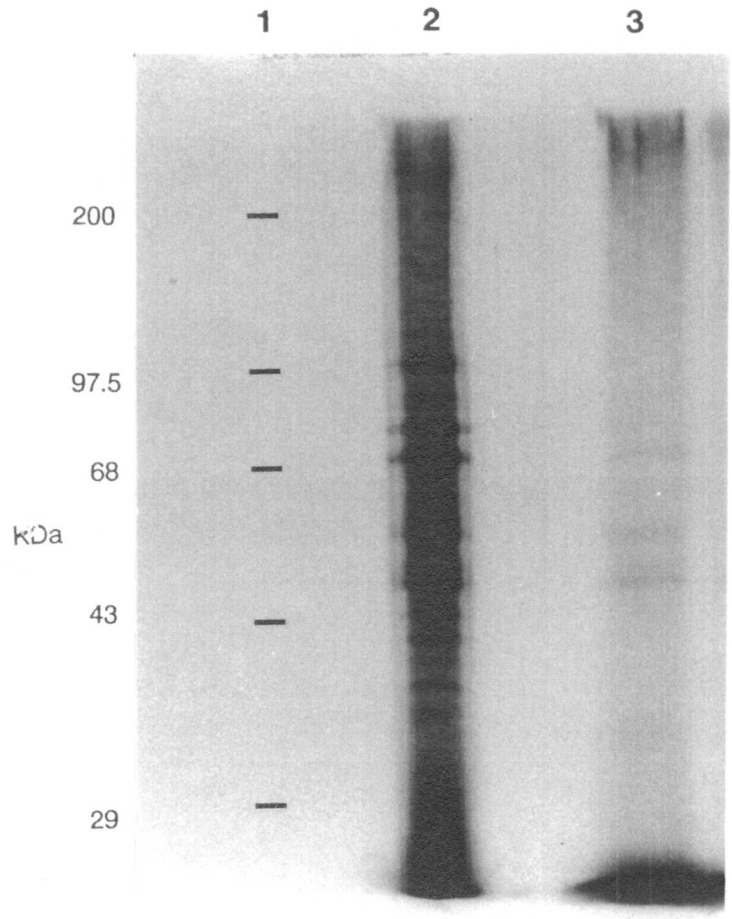


Fig.6.3. A fluorograph showing the effect of Actinomycin D on the production of radiolabelled proteins in non-electroporated *N. tabacum* protoplasts. **Lane 1.** Pre-stained markers (see Fig.6.2) **Lane 2.** Proteins from protoplasts incubated in the absence of Actinomycin D. **Lane 3.** Proteins from protoplasts incubated in the presence of Actinomycin D.

Plate 6.1.

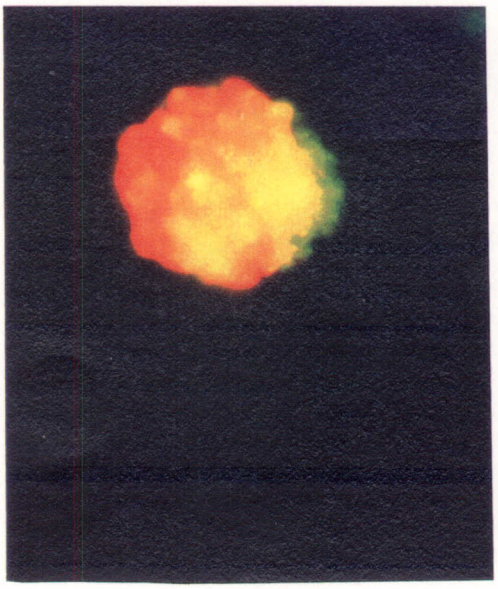


Plate 6.2.

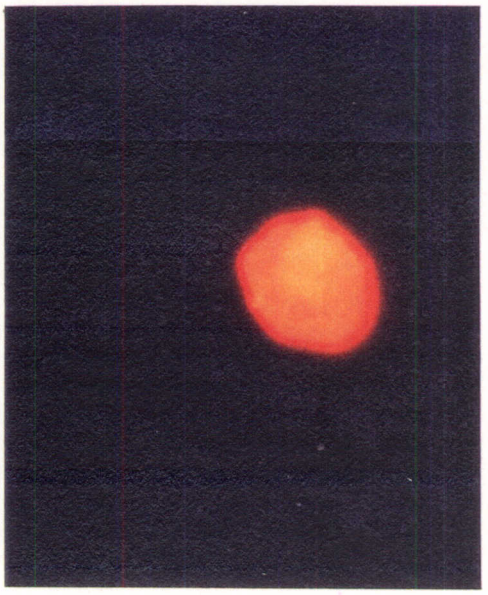


Plate 6.3.

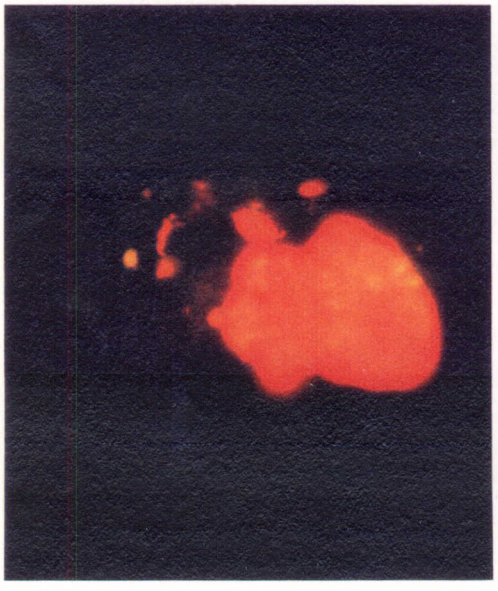
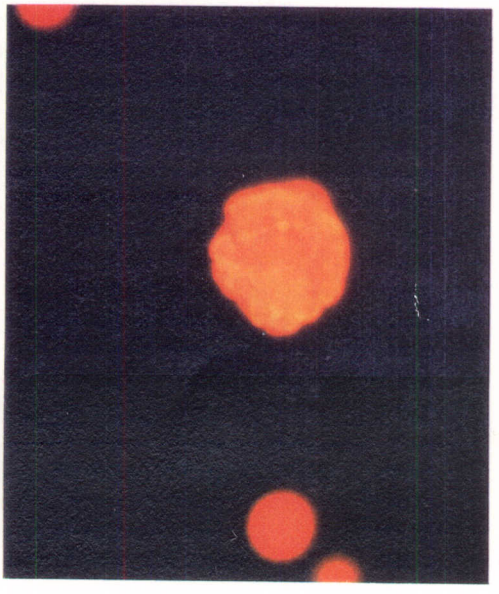


Plate 6.4.



Plates 6.1-6.4. Immunofluorescence of infected and uninfected protoplasts.

Plate 6.1. Protoplast electroporated with PSbMV-RNA. Viral coat protein was detected using antiserum to the PSbMV CP and 2º antiserum conjugated to fluorescein isothiocyanate.

Plate 6.2. Protoplasts mock inoculated and treated with antisera as for Plate 6.1.

Plate 6.3. Protoplasts electroporated with PSbMV-RNA. Viral cylindrical inclusion protein was detected using antiserum to the PSbMV CI protein and 2º antiserum as for Plate 6.1.

Plate 6.4. Protoplasts mock inoculated and treated with antisera as for Plate 6.3.

arrowed as corresponding to the CI protein in lane 3 (b) is seen to have a similar mobility to the band arrowed in lane 4. No band appeared in the MDL translation that corresponded to the band arrowed as representing the AI protein in the protoplasts (c). This is because the AI protein is not found as a mature protein in MDL (Dougherty and Hiebert 1980c: R.E. Rhoads pers comm.). Arrow 'd' indicates a band with a molecular weight corresponding to the NIb protein. It is not clear which band represents the NIb protein in the MDL, so it has not been indicated.

In Fig.6.3, the effect of Actinomycin D on the accumulation of ^{35}S labelled host proteins is seen. The samples comprised uninfected protoplasts which were not electroporated, incubated with (Lane 3) or without (Lane 2) Actinomycin D. Few bands appear in the samples derived from protoplasts incubated with Actinomycin D compared to the samples where Actinomycin D was absent. The Actinomycin D appears to be an efficient inhibitor of host mRNA transcription.

Proteins produced in protoplasts electroporated with PSbMV-RNA and whole virus were analysed using Western blots in an attempt to establish whether the CP of PSbMV had been translated in the protoplasts. The analysis of proteins in electroporated protoplasts using Western blots (Fig.6.4) was partly successful. A faint band (lane 6) that co-electrophoresed with purified coat protein (lane 1) was observed from *N.tabacum* protoplasts electroporated with PSbMV-RNA suggesting that CP may have been produced in these protoplasts. The immunoprecipitation of proteins with coat protein antiserum (Fig.6.4 lanes 2 and 3) showed a band from both samples of *Nicotiana* that co-electrophoresed with the major band of the purified coat protein (Lane 1). The band may represent the CP or alternatively may be an immunoglobulin labelled with 2° antiserum, which is the probable explanation for the appearance of the other bands observed in lanes 2 and 3. Lanes 4 and 5 are the proteins from mock-electroporated controls, and lanes 7 and 8 are the result of the protoplasts electroporated with whole virus. Lane 9 is also a control of mock-electroporated protoplasts. No bands were present in lanes 4, 5, 7, 8, and 9.

Protoplasts electroporated with PSbMV-RNA were analysed for the presence of whole virus particles using electron microscopy. The grids were dipped into a suspension of intact protoplasts. The protoplasts lysed on the grids. Electron microscope examination of immunotrapping grids showed no evidence of virus particles.

Immunofluorescence was used to determine the presence of the CP and CI protein of PSbMV in protoplasts electroporated with PSbMV-RNA. Immunofluorescence (Plates 6.1 to 6.4) revealed differences between the fluorescence exhibited by the electroporated protoplasts and the mock-electroporated controls when PSbMV CP and PSbMV CI 1° antisera were used. Protoplasts seen in Plate 6.1 have been reacted with the PSbMV CP as the 1° antiserum. The green glow of the fluorescing fluorescein isothiocyanate indicates that the CP is present in the cytoplasm of this cell. Plate 6.2 is a mock-electroporated protoplast reacted with PSbMV CP and 2° antiserum. Plate 6.3 shows a protoplast reacted with PSbMV CI as the 1° antiserum. The green colour is more diffuse than in Plate 6.1 because the cell has lysed and the cytoplasm is extruding

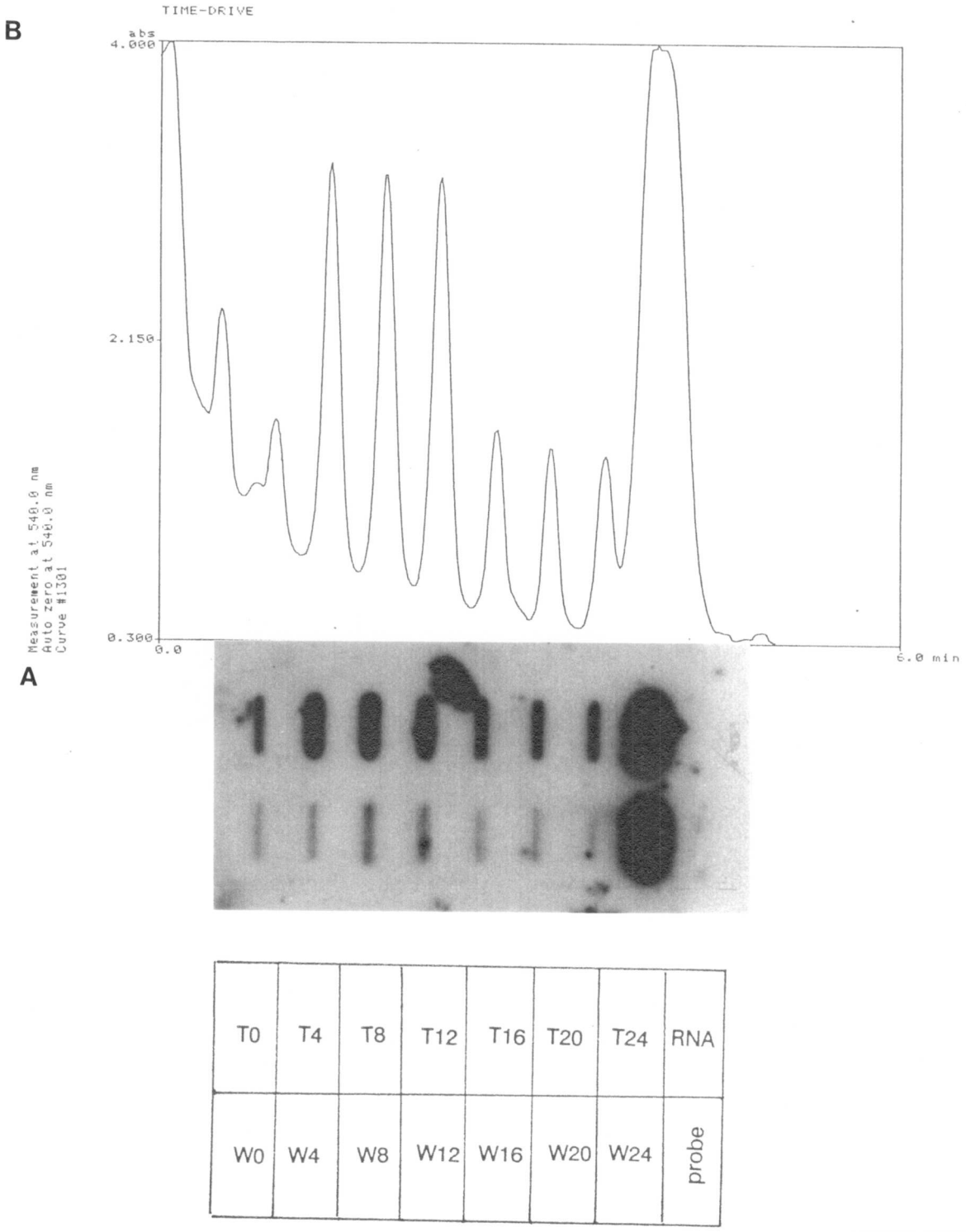


Fig.6.5. A. RNA dot blot analysis of samples taken at set time intervals from *N. tabacum* protoplasts electroporated with PSbMV-RNA. **B.** Peaks of a graph of densitometer readings are shown to correspond to each of the different time intervals.

W= wash removed after appropriate incubation times T= time *N. tabacum* protoplasts were incubated after electroporation with PSbMV-RNA

into the surrounding medium. The green colour does however indicate that this protoplast contains CI protein. Plate 6.4 shows mock-electroporated protoplasts reacted with PSbMV CI and 2° antiserum. Approximately 10% of the protoplasts (estimated visually) showed fluorescence when either PSbMV CP or PSbMV CI 1° antisera were used. As described in Chapter 3, no filters were available to reduce the fluorescence of the chlorophylls a and b. However, the fluorescence was quite bright and the results of the "blind" experiments indicated the technique was reliable.

Fig.6.5 shows RNA dot blot analyses of samples taken at 0, 4, 8, 12, 16, 20 and 24 hour intervals from suspensions of protoplasts electroporated with PSbMV-RNA. Fig.6.5 'A'shows the RNA dot blot of samples taken over a 24 hour period. The signal intensity of RNA is seen to rise for the first 4 hours. The signal then remained constant for the next 8 hours before suddenly decreasing between 12-16 hours. The signal then remained constant for the remainer of the experiment (16-24 hours).

The protoplasts in each sample were washed three times in an attempt to remove any PSbMV-RNA that was loosely associated with the outside of the protoplasts. The RNA dot blot depicted in Fig.6.5A shows the RNA that was present in the final wash of the protoplasts at each of the incubation times. The washes can be seen to follow the pattern produced by the protoplast fraction.

The levels of RNA present in the protoplasts at any one time are indicated by the densitometer readings taken at 540 nm (Fig.6.5 B). The densitometer readings indicate a rapid increase in the amount of PSbMV RNA present in the protoplasts after 4 hours of incubation. The level of RNA increases approximately two fold. The amount of RNA decreased rapidly between 12-16 hours incubation, then remained constant. The large peak observed at the end of the graph represents 10 pg of purified PSbMV RNA.

The local lesion host for PSbMV, *Chenopodium amaranticolor*, was inoculated with protoplasts electroporated with PSbMV-RNA in an attempt to show whether infectious material was present in the protoplasts. Controls of infected pea sap and purified PSbMV-RNA were included. No lesions were observed on the *C.amaranticolor* leaves inoculated with electroporated protoplasts or mock-electroporated controls. However, both the infected pea sap and the purified viral RNA produced lesions. The average number of lesions produced when pea extract was used as inoculum was 3.5 per leaf. PSbMV-RNA also produced an average of 3.5 lesions per leaf. The number of lesions produced by the PSbMV-RNA was small. The most likely explanation is that the RNA underwent degradation because the leaves of the *C.amaranticolor* were not surface-sterilised before the RNA was applied. I considered that surface sterilisation would not necessarily eliminate all the RNases present and might bias the uptake of the RNA by the cells. The low number of lesions observed on the leaves inoculated with pea extract may reflect the low number of virus particles in the sap of infected plants.

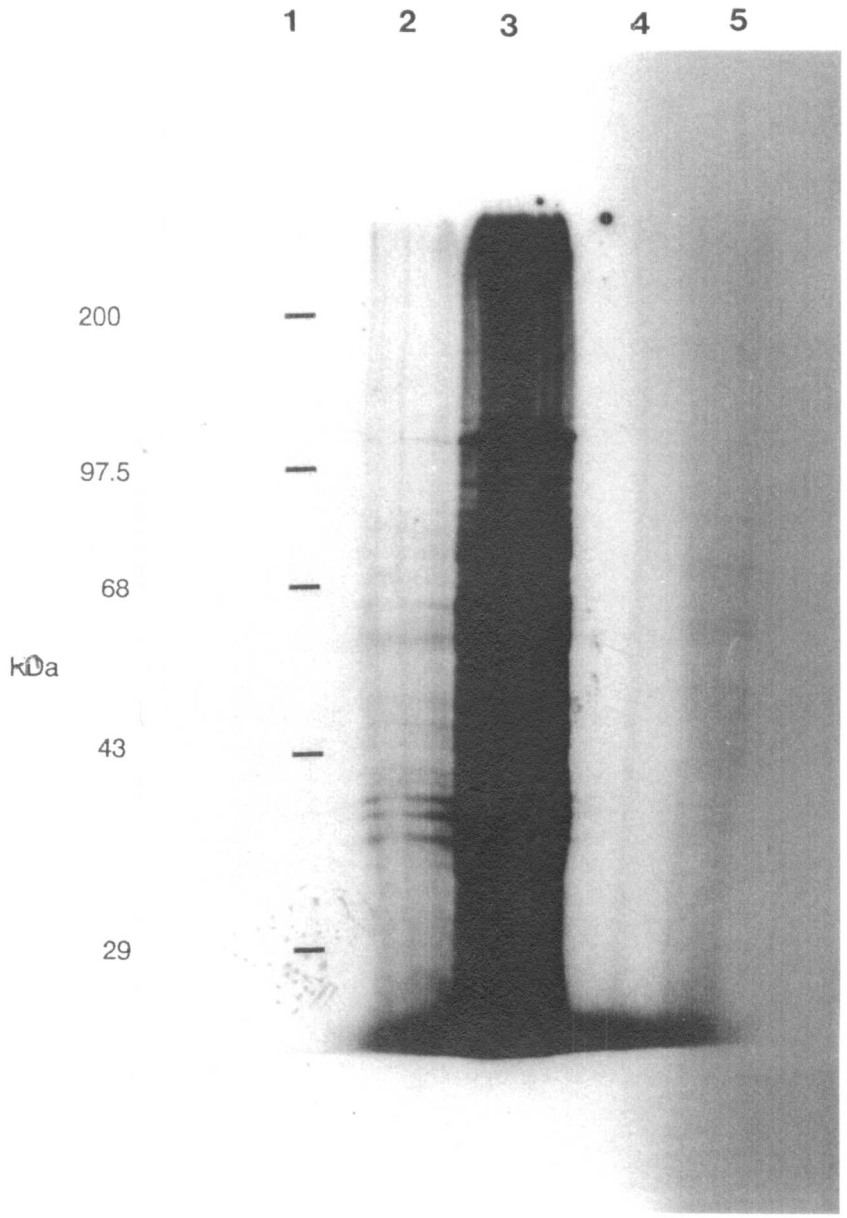


Fig.6.6. ^{35}S labelled proteins produced by protoplasts electroporated with whole virus isolated by different procedures separated on a SDS polyacrylamide gel and detected by fluorography. **Lane 1.** Markers (the same as Fig. 6.2). **Lane 2.** Proteins from a mock electroporated protoplast control. **Lane 3.** Proteins labelled in protoplasts electroporated with virus in infected pea leaf extract. **Lane 4.** Proteins labelled in protoplasts electroporated with partially purified virus. **Lane 5.** Proteins labelled in protoplasts electroporated with whole virus stored at -20°C.

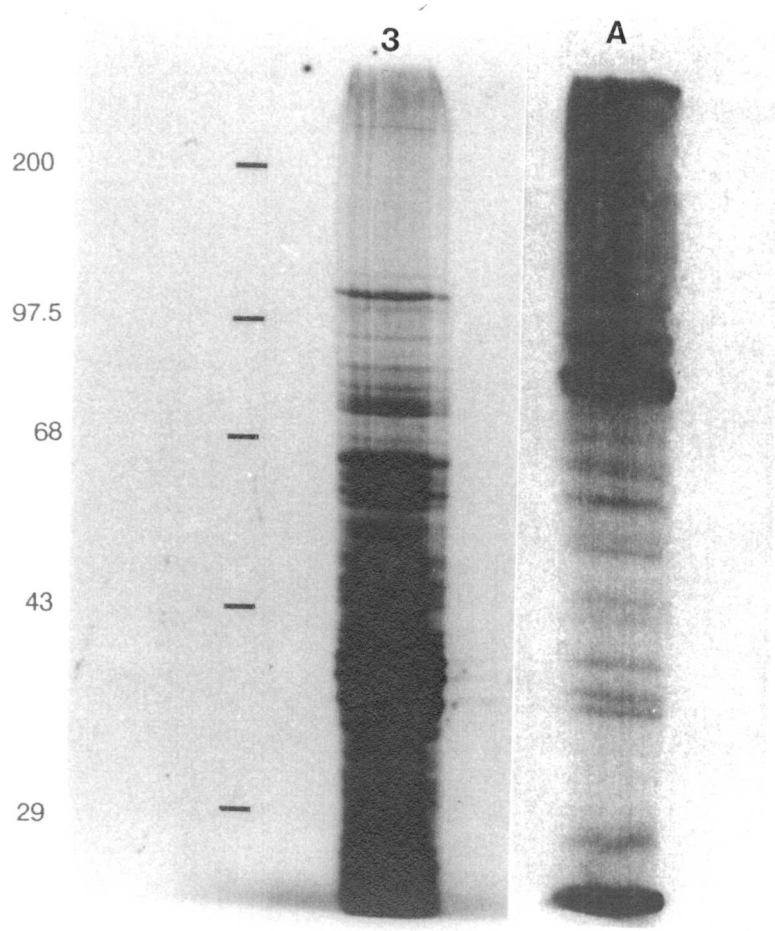


Fig.6.7. The fluorograph is from the same gel as depicted in Fig.6.6 but is exposed to X-ray film for only 3 hours instead of the 12 hours for Fig.6.6. **Lane 3.** Radiolabelled proteins produced by protoplasts electroporated with whole virus. **Lane A.** Radiolabelled proteins produced in rabbit reticulocyte lysate programmed with PSbMV-RNA and exposed to X-ray film overnight. **The position of the markers** (the same as Fig.6.2) **are indicated**. Lanes 2, 4, and 5 from Fig.6.6 are not indicated on Fig.6.7 as the bands were very faint.

6.3.3 Electroporation of Whole Virus

The results of a preliminary study electroporating whole virus from a variety of sources into protoplasts is presented in Fig.6.6. Two different exposures of the gel are presented (Figs.6.6 and 6.7). The fluorograph depicted in Fig.6.6 was over-exposed but a shorter exposure meant that the bands in lane 2 disappeared. In the shorter exposed Fig.6.7, only lane 3 can be clearly seen. Three different sources of inoculum were used: homogenised infected pea leaves (lane 3); partially purified virus (lane 4); and whole virus stored at -20°C (lane 5). The protoplasts electroporated with homogenised, infected pea leaves (lane 3) show a large number of ^{35}S labelled proteins. Many of the radiolabelled proteins were probably derived from cellular mRNA that was electroporated from the pea extract into the protoplasts along with the whole virus. Although Actinomycin D was present in the incubation mix to inhibit transcription of the protoplast mRNA, this would not prevent the translation of either protoplast mRNA that had already been transcibed or pea mRNA electroporated into the protoplasts. Lane 2 (Fig.6.6) represents a control of mock-electroporated protoplasts. Very few host radiolabelled proteins are present in mock electroporated control. The other lanes (4 and 5) which were electroporated with partially purified virus and whole virus stored at -20°C, respectively, did not show any radiolabelled proteins. The solution containing the partially purified virus lysed the protoplasts. When the bands in lane 3 were compared to an *in vitro* rabbit reticulocyte lysate (Fig.6.7), a number of bands appeared to correspond. However, so did a number of bands from the mock electroporated protoplasts. As an uninfected pea-extract control was not, used no conclusions comparing the proteins produced in MDL and in protoplasts electroporated with whole virus can be drawn.

6.4 DISCUSSION

Protoplasts isolated from *Nicotiana* species were chosen for the electroporation experiments for several reasons. First, the *Nicotiana* protoplasts were robust and easily handled. They withstood electroporation well with only a few cells lysing. In contrast, preliminary tests electroporating pea protoplasts indicated that these were very delicate and could not withstand more than 80 V and 330 µF without more than 70% lysis of the cells (data not presented). At these low voltages, it was uncertain whether the permeability of the plasma membrane would be altered enough to allow the viral RNA to enter the cell. Moreover, a cell that appears to be this delicate is likely to suffer greater stress during electroporation (at the same voltage) than "tougher" cells. It may take longer for pea protoplasts to regain normal metabolism, if they ever do. Finally, there is a large body of literature outlining various techniques to introduce both whole virus and viral RNA into protoplasts of *Nicotiana* species that is not available for pea protoplasts.

The current study attempted to establish whether Nicotiana species could be infected with PSbMV. Various tests showed that N. tabacum and N. clevelandii were not able to function as

host species for PSbMV. No visible symptoms were evident on the *Nicotiana* plants during the 6 weeks of observation following inoculation. The immunotrapping grids, the ELISA test and the RNA dot blots all indicated that PSbMV particles were unable to establish a detectable infection in the two species of *Nicotiana* examined. These results support the list of host plants given in the CMI/AAB, where *Nicotiana* species are excluded (Hampton and Mink 1975).

The infection of the local lesion host *C. amaranticolor* with inoculum from electroporated protoplasts produced no lesions. It is assumed that the levels of inoculum were too low to induce lesions, as SDS-PAGE and fluorography, Western blots, immunoprecipitation of viral CP, and RNA dot blots all indicated that viral replication and translation took place in the protoplasts. Viral RNA (used as a positive control) is reported here to infect *C. amaranticolor*. Infection by naked PSbMV-RNA has not previously been reported. Other potyviral RNA has been shown to be infectious, for example wheat streak mosaic virus (Brakke and van Pelt 1970).

The *Nicotiana* protoplasts electroporated with PSbMV-RNA were able to translate viral RNA to produce proteins that correspond in size to viral proteins. In the fluorograph depicted in Fig.6.2, bands were observed in the inoculated lane that were not present in the mock-inoculated control. These bands, with estimated molecular weights of 86, 70, 54, and 48 kDa, may represent the 86 kDa, CI, AI, and the NIb proteins respectively. It is interesting to note the possible presence of the AI protein. This protein was not detected in the rabbit reticulocyte lysate translations (see Fig.5.7 and Chapter 5 for discussion). The 86 kDa major band appearing in the rabbit reticulocyte translations was also present as a band in the protoplasts electroporated with PSbMV-RNA. It is therefore possible, that the final processing of the 86 kDa precursor is delayed in both protoplasts and MDL programmed with PSbMV-RNA. A discussion of a possible role for precursors was given in the preceding chapter.

Autoradiography has been used by several workers to detect virus directed translation products in protoplasts (Watanabe *et al.* (1984), Sakai *et al.* (1977), and Rottier *et al.* (1980)). While most workers rely upon differences observed between the bands produced by virus treated and mock treated samples, this alone does not establish the viral origins of the proteins. Of the workers cited above, only Rottier *et al.* (1980) used an immunoprecipitation procedure to establish the viral origins for one of the proteins.

The addition of Actinomycin D helped to clarify the picture both in my study and in those of other workers. Any reduction in the number of host proteins labelled with [^{35}S] methionine simplified determination of which proteins were of host origin and which were of viral origin. A reduced amount of host-mediated protein (estimated by the visual intensity of the bands) was observed in my study when Actinomycin D was present during the incubation period. Rottier *et al.* (1979) similarily achieved a 90% reduction in host-dependent RNA synthesis without inhibiting CPMV protein synthesis. Watanabe *et al.* (1984), working with TMV, also experienced a reduction in the amount of host proteins synthesised.

Western blots were also used to demonstrate the viral origins of the proteins observed in my study. Western blot analyses of viral coat protein production by the protoplasts electroporated with PSbMV-RNA indicated a band that co-electrophoresed with the purified coat protein of PSbMV. The band was faint and it can only be assumed that the production of coat protein was at levels that are just detectable using the Western blot system (see Chapter 4). An effort to improve on this result by immunoprecipitating the coat protein prior to Western blotting was made. A band that co-electrophoresed with purified coat protein was observed, however, this band could be due to immunoglobulin fragments labelled with 2° antiserum.

Immunofluorescence microscopy was the second method used to determine the presence of viral proteins in RNA electroporated protoplasts. The results using antisera to the CI and the CP of PSbMV indicated that protoplasts electroporated with viral RNA translated the RNA into viral proteins (at least CP and CI protein). Other workers, for example Maule (1983); Hibi *et al.* (1975) and Aoki and Takebe (1969), used immunofluorescence successfully to detect the presence of a variety of viruses in their protoplast systems. Problems relating to the unavailability of filters to remove the fluorescence of the chlorophylls have been discussed in Chapter 3.

The electroporation of whole virus into protoplasts using homogenised infected peas as the source of whole virus produced a large number of proteins. Comparison of the proteins produced by electroporated protoplasts and by *in vitro* translation of PSbMV-RNA revealed many bands with similar molecular weights. However, with so many bands it was difficult to establish with confidence how many virus-specific protein bands were present and how many bands were the result of mRNA from the pea extract being translated in the protoplasts. Several bands are candidates for the coat protein having molecular weights of approximately 33 kDa. However, because of the number of bands present, it was difficult to establish which band might represent the coat protein even with co-electrophoresing purified coat protein in an adjacent lane.

An attempt was made to monitor the accumulation of PSbMV RNA by dot blot hybridisations. In the RNA dot blots samples were removed at 4 hour intervals over a period of 24 hours. The RNA level was observed to increase about two fold for the first 4 hours and then remain constant for 8 hours before decreasing between 12 and 16 hours. After this time the level of RNA remained constant. The final wash solution of the protoplasts followed the same pattern of increasing then decreasing amounts of RNA. It is well documented that protoplasts retain virus particles on their outer surfaces (Maule 1983; Roenhorst *et al.* 1988) and it is possible that RNA is trapped in a similar manner. The three washes preceding the blotting experiment were used to remove any RNA that might be loosely associated with the protoplast surface. As the amount of RNA in the washes follows that of the RNA associated with the protoplasts, it is difficult to say whether this represents RNA retained on the outside of the protoplasts or whether it is likely to be due to a few protoplasts lysing during the final wash, releasing the accumulated RNA into the

wash solution. This explanation would account for the parallel increases and decreases observed between the wash solution and the protoplasts.

Maule (1983) used DNA dot blots to study the amount of CaMV-DNA accumulating in protoplasts isolated from several different hosts. The patterns of DNA accumulation appeared to depend upon the host species, with the time taken for viral DNA synthesis to reach optimal levels varying considerably. Optimal transcription of CaMV was reached in 24-48 hours in *Brassica rapa* protoplasts. Other viruses have also been studied. Maule *et al.* (1980b) used plant assays to test the replication of cucumber mosaic virus (CMV) in cucumber protoplasts. The technique consisted of extracting the RNA from the infected protoplasts and then inoculating cowpea leaves with the RNA. The cucumber mosaic virus was found to reach maximum replication between 38 and 40 hours. Harrison *et al.* (1976) also used infectivity assays to determine that infective tobacco rattle virus (TRV) was produced after 11-12 hours. In a study involving two potyviruses (PVY and TVMV), Luciano *et al.* (1987) electroporated *N. tabacum* protoplasts with 80 μg of viral RNA. Replication of viral RNA was measured by molecular hybridisation to ^{32}P-TVMV DNA in a quantitative dot blot assay. They detected increases in TVMV RNA concentration 40 minutes after electroporation, with the most rapid rate occuring from 3-15 hours after electroporation. After 15 hours incubation the amount of TVMV RNA had increased approximately three fold. In my study, molecular hybridisation techniques were also used to monitor the level of RNA in the electroporated protoplasts. The level of PSbMV RNA increased most rapidly during the first 4 hours after electroporation with an approximately two fold increase observed for PSbMV RNA during this period. This observation is similar to the three fold increase observed for TVMV during 4-12 hours incubation. It is notable that the two potyviruses cited and PSbMV reached a maximum rate of replication much faster than CaMV and CMV, and marginally faster than TRV. This may be one reason why potyviruses are so successful as plant pathogens (Milne 1988). It must be a considerable advantage to any pathogen to reach maximum replication before the plant's defence system can be induced.

In addition to out manouvering the plants defense system, rapid replication carries several other advantages. When infecting annuals in particular (as many potyviruses do), rapid replication, translation, and the assembly of virus particles may decrease the time taken to initiate cell-to-cell spread and ultimately a systemic infection. The low concentration of potyvirus particles observed in sap (Stevenson and Hagedorn 1973), compared to the large amounts of viral products that accumulate as inclusions in cells infected with potyviruses (de Mejia *et al.* 1985a, 1985b; Dougherty and Hiebert 1980b) , suggests that potyvirus particles maybe disseminated quickly. The speed with which intact virions are assembled is also directly related to the time elapsed between initial infection and the stage at which the virus is able to be spread to another plant by its aphid vector. A final point for consideration is that the speed with which

replication is established must render potyviruses formidable competitors against other plant viruses.

A further point pertinent to the success of potyviruses as pathogens was briefly discussed in the preceding chapter. The organisation of the potyvirus genome includes several features that may help make this group competitive. These features, the 3' and 5' untranslated sequences, the VPg and the poly[A] tail, were discussed in the previous chapter as being potentially important translation enhancers. Plant viruses, unlike animal viruses, are reputed not to "turn off" their host machinery. This means that they must compete with the host mRNA for ribosome binding sites. If, as suggested by Gallie *et al.* (1989), plant viruses contain highly efficient translation enhancers, their competition for ribosome binding sites becomes more successful. Herson *et al.* (1979) found that TMV RNA was preferentially translated in *in vitro* competition assays. This may reflect the ability of the virus to capture cellular translation machinery efficiently once it has entered the host cell (Gallie *et al.*1987; Plaskitt *et al.* 1988).

In summary, the protoplasts that were derived from a non-host plant (*Nicotiana*) and electroporated with PSbMV-RNA were able to transcribe and translate the viral RNA. The infection of the local lesion host, *C. amaranticolor*, with PSbMV-RNA establishes that the RNA of PSbMV is infectious. In the light of the success of the RNA dot blots, it would appear that clone pPSb70 is a good probe for RNA hybridisation assays of infected plant material.

CHAPTER 7

REVIEW AND PROSPECTS

Our understanding of PSbMV at a molecular level has been extended by this study.

The first problem facing a plant virus study is to perfect a technique to purify the virus in question. For PSbMV, extensive modifications to the procedure of Reddick and Barnett (1983) were required to purify PSbMV from peas. The modified method resulted in good yields of predominantly full length particles. The isolation of full length PSbMV-RNA was also a difficult task. Good yields of predominantly full length RNA were obtained using the method of Brakke and van Pelt (1970). Some of the RNA was used to produce a cDNA library of 360 clones. One of these clones, pPSb70, was used as a probe for detecting PSbMV in infected material. This clone has potential as a probe in nucleic acid hybridisations. The technique of nucleic acid hybridisation is attracting considerable attention as an alternative to the ELISA test for detecting virus in infected material. This technique is especially relevant for PSbMV as the ELISA test can produce unreliable results for PSbMV (A.Russell pers. comm.).

"Genetically engineered" cross-protection has revolutionised the concept of 'virus-resistant' crops. The resistance of peas to infection by PSbMV is controlled by a single recessive gene (gene *sbm*) and considerable time and effort is required to breed resistance into new varieties of peas. The prospect of producing "genetically engineered" crops with improved resistance to PSbMV is another use for cDNA clones. Clone pPSb70 is a suitable candidate for this programme. Other clones present in the library, but not yet characterised, may also be suitable. In addition, the production of cDNA using an oligo-(dT) primer and the subsequent sequencing of pPSb70 (Appendix A) have helped to confirm that the genome of PSbMV is polyadenylated at the 3' end. This has been observed for other potyviruses, for example TEV (Allison *et al.* 1986).

A fundamental question about a virus is the nature and molecular weight of the proteins it encodes. The only previously established molecular weight for a PSbMV-encoded protein was the CP with a molecular weight of 34 kDa (Huttinga 1975). My study establishes the molecular weights of 3 mature virus-encoded proteins using Western blots. The molecular weight of the CP was established to be 33 kDa by both Western blots, SDS-PAGE and immunoprecipitation of *in vitro* translation products with PSbMV CP antiserum . The molecular weights of the CI and NIb proteins (70 and 48 kDa respectively) were also established by Western blot analysis and confirmed by immunoprecipitation of *in vitro* translation products. The molecular weight of the NIa protein (45 kDa) was established by immunoprecipitation of *in vitro* translation products with TEV NIa antiserum. The suggested molecular weight for the AI of PSbMV (54 kDa) drew on the published estimates of the AIs from other potyviruses to decide which of the four bands observed

in the Western blots was the most likely candidate for the precursors to the AI of PSbMV. The cylindrical inclusion and coat proteins of PSbMV were observed to have similar molecular weights to their counterparts in TEV and TVMV (Dougherty and Carrington 1988). The two nuclear inclusion proteins of PSbMV had slightly different molecular weights to those observed for either TEV or TVMV. The molecular weights of the NIa and NIb proteins of TEV, TVMV and PSbMV are all different. The two proteins flanking the AI proteins were not observed directly but are calculated to have molecular weights of 32 and 42 kDa. Only the molecular weight of the VPg remains to be determined for PSbMV.

The data obtained from *in vitro* translations of PSbMV RNA are consistent with the production of a polyprotein which is cleaved into 7 or more mature proteins. This strategy has been presented for other potyviruses, for example SMV (Vance and Beachy 1984b). TEV and TVMV have been shown to produce a single polyprotein which, if uncleaved, would have molecular weights of 346 kDa and 340 kDa, respectively. (Allison *et al.* 1986; Domier *et al.* 1986). The suggestion that the CP is encoded on a sgRNA has been presented several times as a possible strategy for potyviruses (Otal and Hari 1983; Dougherty *et al.* 1985) However, the detection of only full length PSbMV-RNA isolated from the polyribosomes of infect pea leaves supports the weight of evidence against this strategy. The gene map proposed for PSbMV in Chapter 5 presents an organisation similar to the maps proposed for several other potyviruses for example BYMV (Chang *et al.* 1988). The coat protein gene is confirmed as being present at the 3' end of the genome. The position of this gene has been the subject of considerable speculation.

The infectious nature of some potyvirus RNA, for example that of wheat streak mosaic virus, has been established (Brakke and van Pelt 1970). Although PSbMV has been used to mechanically inoculate plants for more than a decade, the infectious nature of its RNA has not previously been reported. The production of local lesions on the host *Chenopodium amaranticolor* inoculated with purified RNA establishes the infectious nature of PSbMV-RNA.

The production of PSbMV RNA in protoplasts was examined using Northern blots. The blots indicated that the amount of PSbMV-RNA in electroporated protoplasts increased very rapidly for the first 4 hours after electroporation and was maintained for 12 hours. After this, the amount of RNA was seen to decline. The speed with which replication of potyvirus RNA is initiated and maintained is suggested in Chapter 6 to be one of the reasons that potyviruses are so successful as pathogens. Incorporation of ^{35}S methionine indicated that PSbMV-RNA was translated in *Nicotiana tabacum* protoplasts.

The directions that future studies in plant virology and especially the biology of potyviruses may take are varied. One of the last major areas to be elucidated is the interaction between the virus and its host. Such questions as how the viruses cause disease symptoms, and what effects viral proteins have on normal plant physiology are attracting much attention. Another area of interest is the response of plants (both host and non-host) to the presence of different viral

proteins. Answering such questions may help determine the reason for restricted host range and possibly the mechanism of "genetically engineered" cross-protection. The mechanism whereby the Al protein of potyviruses is used to aid the uptake of virus by its aphid vector also remains unsolved.

Other areas that may be examined include the functions of unprocessed precursor molecules. The apparently delayed processing of the 32K+Al precursor of potyviruses suggests that this precursor has a function. As discussed in Chapter 5, a precursor of cowpea mosaic virus is responsible for RNA chain elongation (Dorssers *et al.* 1984). Several functions that would seem to require a protein do not as yet have one assigned. Among these is the separation of the negative strand of RNA from the positive strand after replication. One may suggest that a protein, either virus-encoded or host-mediated, is responsible for this act.

Sequence data provide us with a great deal of information which may be used to elucidate other aspects of the behaviour of viruses. Comparison of the inferred amino acid sequence for potyvirus proteins with those from viruses of other groups may allow us to assign functions to some of the potyvirus encoded proteins. Domier *et al.* (1987) have already assigned a putative function as a replicase to the NIb protein from TEV, based on sequence similarity with a protein of known function from other viruses.

Finally, nucleotide sequence data may be useful to observe the development of new virus strains. RNA viruses have mutation rates up to a million times higher than DNA genomes (Lloyd 1986; Reanney 1982). The capabilities of reassortment and recombination within their RNA genomes presents these viruses with the opportunity to adapt to a variety of ecological niches in a short period of time (Turpen, in press). This evolutionary plasticity presents formidable disease problems, making RNA viruses some of the most successful parasites known.

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Appendix A

Nucleotide Sequence of the Coat Protein Gene of Pea Seed-borne Mosaic Virus Pathotype P-1

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SUMMARY

The nucleotide sequence of a 1355 base pair cDNA representing the 3'-terminal sequences of pea seed-borne mosaic virus (PSbMV) was determined. This sequence contained a single long open reading frame (ORF) of 1189 base pairs ending with a single TAA termination codon. Downstream from the ORF was an untranslatable region of 189 base pairs followed by 8 base pairs of polyadenylate. The probable location of the PSbMV coat protein codons within the long ORF was determined by comparing the inferred amino acid sequence with other potyviral coat protein sequences and by examining the sequence for a potyviral polyprotein cleavage cassette sequence. Direct chemical sequencing of the PSbMV coat protein revealed it to be blocked at its amino terminus. A minority sequence representing the N terminus of the protease-resistant core of the coat protein was determined, however. Alignment of the PSbMV coat protein sequence and the sequences of seven other potyviral coat proteins revealed significant homology; ranging from 53.7% for potato virus Y strain D to 43.2% for tobacco vein mottling virus.

The potyviruses are reputed to be the largest group of plant viruses, and many cause economically significant disease. Potyviruses have filamentous virions which carry a single stranded, positive sense RNA genome of approximately 10,000 nucleotides. Their RNA genome is covalently modified at its 5' end by a virus-encoded protein (VPg) and has a 3' polyadenylate tail. The complete nucleotide sequences of three potyviruses, tobacco etch virus (TEV) (Allison *et al.*, 1986), tobacco vein mottling virus (TVMV) (Domier *et al.*, 1986) and plum pox virus (PPV) (Maiss *et al.*, 1989) have been determined. Amino acid and/or nucleotide sequences have also been determined for a number of other potyviral coat proteins or their genes.

Pea seed-borne mosaic virus (PSbMV) is an important member of the potyviral group of plant pathogens. It infects a variety of plant species, with the greatest economic impact occurring when peas (*Pisum sativum*) are infected (Hampton & Mink, 1975). The virus has a broad geographic distribution and has probably been spread throughout the world by infected seed. Aspects of the biology and epidemiology of PSbMV have been reviewed recently (Khetarpal & Maury, 1987).

Recently, transgenic plants expressing plant viral coat protein genes have been shown to be less susceptible to viral disease, a phenomenon termed "genetically engineered cross-protection" (Nelson *et al.*, 1987). Determination of the sequence of the PSbMV coat protein gene is the first step toward producing transgenic plants expressing this gene, and ultimately toward using this technology to protect grain legume crops from PSbMV caused disease. This paper reports the nucleotide sequence of the 3'-terminal 1355 nucleotides of PSbMV, which includes the entire coat protein coding region.

Pea seed-borne mosaic virus, pathotype P-1, was purified from infected *Pisum sativum* plants using a modification of the procedure published by Reddick & Barnett (1983). Full-length viral RNA was isolated from freshly prepared virus as described by Brakke and van Pelt (1970). Complementary DNA was synthesized by the single tube reaction described by D'Alessio, *et al.* (1987) using an oligo $(dT)_{12-18}$ primer, then cloned into SmaI-digested, dephosphorylated pUC19 plasmid using standard methods (Maniatis *et al.*, 1982). The resulting library contained 360 clones and was screened for the length of the inserted cDNA by digesting mini-preparations of plasmid DNA (Birnboim & Doly, 1979) with restriction endonucleases EcoR1 and BamH1. Three clones, pPSB70, pPSB67 and pPSB13, containing inserts of 1,355 and approximately 1,270 and 1,000 base pairs respectively were chosen for preliminary nucleotide sequence analysis. Restriction fragments from these clones were subcloned into the polylinker region of M13mp18 or -mp19, and DNA sequences were determined by the dideoxy chain termination method of Sanger *et al.* (1977).

All three of the clones examined contained 3' poly(A) tracts as well as identical sequences adjacent to the polyadenylate tail. The complete nucleotide sequence of the 1355 base pair cDNA inserted in plasmid pPSB70 was determined. The resulting sequence was assembled and analyzed on an IBM-PC compatible computer using the GENESYS software written by W. Bottomley (CSIRO Division of Plant Industry, Canberra, Australia).

The sequence of the 1355 nucleotides at the 3'end of PSbMV genomic RNA is presented in Figure 1. This sequence contains a single long open reading frame (ORF) found on the (+) strand and ending with a single termination codon (TAA) at nucleotide 1189. It has a 3'-untranslatable

region of 159 nucleotides and ends with eight adenylate residues. The sequence is purine rich, containing 33.5% adenosine and 26.3% guanosine, as well as 22.9% thymidine and 17.3% cytidine

Computer analysis of the sequence has revealed a number of direct repeats as well as regions which may form stable stem-loop structures. In the 1355 nucleotide long sequence there are nine pairs of identical nonanucleotides and a single pair of undecanucleotides (data not presented). Sequences that might form regions of RNA secondary structure have been predicted by applying the rules of Tinoco *et al.* (1973). A number of stem-loop structures can be predicted for the entire sequence, with the most stable stem structure having a ΔG value of -16.6 (data not presented). The 3' untranslated region may be relatively free of RNA secondary structure since no stem structures in this region are predicted to have free energies less than -10.0.

The single ORF of 1189 nucleotides is long enough to encode the PSbMV coat protein as well as some of the preceding cistron. No other extended ORF is found by computer analysis of either the (+) strand or the (-) strand. Like other potyviruses, the primary translation product of the PSbMV genome is probably a polyprotein which is proteolytically cleaved to produce the mature viral proteins (Calder, 1989). As is the case with TEV, TVMV and PPV, the codon that initiates translation is expected to reside near the 5' end of PSbMV genomic RNA, and therefore is not present in the sequences reported in this paper.

The primary translation products of other potyviruses are proteolytically cleaved at either Q-A, Q-S or Q-G dipeptides to release

mature coat protein. The probable amino-terminus of the PSbMV coat protein has been predicted by examining the amino acid sequence of the long ORF. The most likely precursor cleavage site for the coat protein is between the Q-A dipeptide found at nucleotides 325-330 (Fig. 1). The amino acid sequence at this site (V-R-L-Q-A) closely resembles the consensus cleavage sites described for TVMV polyprotein processing (Domier *et al.*, 1986) as well as the cleavage sites used by other potyviruses, except for TEV (Dougherty *et al.*, 1989). Cleavage at this site produces a coat protein of 287 amino acids, with a calculated molecular mass of 32651 Da. This agrees well with the results of SDS-polyacrylamide gel electrophoresis of purified PSbMV, which produces two protein bands having mobilities corresponding to 36 and 33 kDA (Calder, 1989). It is common to observe heterogeneity in the size of potyviral coat proteins, and this is often due to proteolytic removal of the hydrophilic N-terminal domain.

Direct chemical amino acid sequencing of the PSBMV coat protein was carried out on 200 pmol of purified whole virus. The sequence of the N-terminus could not be determined directly, probably due to an acetylated amino acid. A minority amino acid sequence representing 6.5% of the total sample was determined, however. This sequence is *-D-*-D-V-D-A-G-S-*-G-*-I-*-V-P, where * represents residues which could not be identified positively. The location of this sequence is identified by the boxed region in Figure 1, and it coincides with the N-terminus of the trypsin resistant core protein described for other potyviral coat proteins (Shukla *et al.*, 1988a). Other potyviral coat proteins have blocked N-termini, including Johnson grass mosaic virus (JGMV) (Gough *et al.* 1987), the highly aphid transmissible strain of TEV (Allison *et al.*, 1985) and PPV (Lain *et al.*, 1988).

An alignment of the PSbMV coat protein sequence with those of seven other distinct members of the potyvirus group is presented in Figure 2. This alignment shows that the PSbMV coat protein has extensive sequence similarity to other potyviral coat proteins throughout its middle and C-terminal regions but, like other potyviruses, has little similarity in its N-terminal region. The amount of homology between the entire PSbMV coat protein and other potyviral coat proteins varies from 53.7% amino acid sequence identity with potato virus Y strain D (PVYD) to 43.2% with TVMV. The percent sequence identity between these proteins is greater when only their middle and C-terminal regions are compared. The homology between the 238 C terminal amino acids of PSbMV and the aligned amino acids from the other seven potyviral coat proteins in Fig. 2 varies from 65.1% for PVYD to 52.5% for TVMV. The PSbMV coat protein gene has a deletion of the codons encoding the sequence M-P-R-Y (amino acids 244 to 247 in Fig. 2). This sequence has been observed in all other potyviral coat proteins sequenced to date. The nucleotide sequence for this region is identical for all three of the PSbMV cDNA clones examined, therefore the deletion of these four codons is unlikely to represent a cloning artifact or an anomaly occurring during cDNA synthesis.

Experiments are currently underway to genetically engineer the PSbMV coat protein coding region so that it is expressed in transformed plants. In the long term, peas will be transformed with the gene and plants expressing coat protein will be tested for susceptibility to viral infection. If successful, plants transformed with this gene will provide valuable germplasm for plant breeders developing PSbMV resistant cultivars of susceptible crops.

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Figure 1. Nucleotide sequence of the 3'terminus of PSbMV RNA. The amino acid sequence predicted for the ORF is presented below the nucleotide sequence. An arrow indicates the probable proteolytic cleavage site for release of coat protein from the polyprotein translation product. The boxed region indicates the amino acid sequence determined by automated Edman degradation. The single termination codon at the end of the ORF is indicated by an asterisk.

Figure 2. Alignment of the amino acid sequences of eight potyviral coat proteins. Amino acids identical in all sequences are boxed. Literature references not cited in the text are: WMV-2, watermelon mosaic virus (Yu *et al.*, 1989); PWVTB, passionfruit woodiness virus strain TB (Shukla *et al.*, 1988b); PVYD, potato virus Y strain D (Shukla *et al.*, 1988c), TEVN, tobacco etch virus non-aphid transmissible (Allison *et al.*, 1985).

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1     GAA GAA CGA ATT GTT GCA ATT TTG GAA TGG GAT AGA AGT AGA GAA TTT TCA CAT AGG CTT GAT GCC ATA TGT GCA   75
      E   E   R   I   V   A   I   L   E   W   D   R   S   R   E   F   S   H   R   L   D   A   I   C   A

76    GCA ATG ATC GAA GCT TGG GGT TAC GAC GAG CTT TTG CAG CAT ATT CGG AAA TTC TAT TAT TGG TTG TTA GAA CAG  150
      A   M   I   E   A   W   G   Y   D   E   L   L   Q   H   I   R   K   F   Y   Y   W   L   L   E   Q

151   GAA CCA TAC AGG AGC ATA GCT CAG GAA GGA AAA GCA CCA TAC ATC GCA GAG ACA GCG CTT CGG CAC CTG TAC ACA  225
      E   P   Y   R   S   I   A   Q   E   G   K   A   P   Y   I   A   E   T   A   L   R   H   L   Y   T

226   AAT GCC ATG GCA ACA CAA AGT GAA CTT GAG AAA TAC ACG GAA GCA ATC AAT CAG CAT TAC AAT GAT GAA GGT GGT  300
      N   A   M   A   T   Q   S   E   L   E   K   Y   T   E   A   I   N   Q   H   Y   N   D   E   G   G

301   GAT GGA TCA ATC AAG GTT CGA TTG CAA GCT GGT GAC GAA ACC AAG GAT GAT GAA AGA AGA AGG AAA GAG GAG GAG  375
      D   G   S   I   K   V   R   L   Q   A   G   D   E   T   K   D   D   E   R   R   R   K   E   E   E

376   GAC AGA AAG AAA AGA GAG GAG AGT ATC GAT GCG AGC CAG TTT GGT TCG AAT CGT GAC AAT AAG AAA AAC AAA AAT  450
      D   R   K   K   R   E   E   S   I   D   A   S   Q   F   G   S   N   R   D   N   K   K   N   K   N

451   AAA GAG AGT GAC ACA TCA AAC AAA TTA ATA GTG AAG TCT GAT CGA GAT GTT GAT GCA GGA TCT TCA GGC ACA ATC  525
      K   E   S   D   T   S   N   K   L   I   V   K   S   D   R   D   V   D   A   G   S   S   G   T   I

526   ACA GTA CCA AGG CTT GAA AAG ATC TCA GCA AAG ATT AGG ATG CCA AAA CAC AAA GGC GGA GTG GCT ATC AGC TTG  600
      T   V   P   R   L   E   K   I   S   A   K   I   R   M   P   K   H   K   G   G   V   A   I   S   L

601   CAA CAT TTA GTT GAT TAC AAT CCA GCA CAA GTT GAC ATT TCA AAC ACT CGA GCA ACG CAG AGC CAG TTC GAT AAC  675
      Q   H   L   V   D   Y   N   P   A   Q   V   D   I   S   N   T   R   A   T   Q   S   Q   F   D   N

676   TGG TGG AGG CGA GTG TCG CAA GAG TAC GGG GTT GGA GAC AAT GAA ATG CAA GTT TTG GCA AGT GGT TTG ATG GTA  750
      W   W   R   R   V   S   Q   E   Y   G   V   G   D   N   E   M   Q   V   L   A   S   G   L   M   V

751   TGG TGC ATT GAA AAT GGA ACA TCG CCT AAC ATA AAT GGG ATG TGG ACA ATG ATG GAC GGG GAA GAG CAG GTT GAG  825
      W   C   I   E   N   G   T   S   P   N   I   N   G   M   W   T   M   M   D   G   E   E   Q   V   E

825   TAC CCC CTA AAG CCA GTG ATG GAT AAT GCG CGT CCA ACT TTC AGA CAG ATA ATG GCG CAT TTC AGT GAC GTA GCG  900
      Y   P   L   K   P   V   M   D   N   A   R   P   T   F   R   Q   I   M   A   H   F   S   D   V   A

901   GAG GCG TAC ATT GAA AAG AGA AAC TCA ACA GAG GTG TAC GCT CTA CAA CGC AAT TTA AGG GAC CCG AGT CTT GCA  975
      E   A   Y   I   E   K   R   N   S   T   E   V   Y   A   L   Q   R   N   L   R   D   P   S   L   A

976   AGA TAT GGT TTC GAC TTC TAC GAA ATC ACA GCA AAG ACA CCT GTG AGG GCA AGA GAG GCA CAC TTT CAG ATG AAA 1050
      R   Y   G   F   D   F   Y   E   I   T   A   K   T   P   V   R   A   R   E   A   H   F   Q   M   K

1051  GCA GCA GCA ATC AGA GGA AAA TCC AAT AGC CTA TTT GGC TTG GAT GGG AAC GTT GGG ACA CAG GAG GAG AAC ACG 1125
      A   A   A   I   R   G   K   S   N   S   L   F   G   L   D   G   N   V   G   T   Q   E   E   N   T

1126  GAG AGG CAC ACA GCA GAA GAT GTC AAT CAG AAT ATG CAC AAT CTT CTC GGA ATG AGA GCC ATG TAA TCCGTATGTAT 1202
      E   R   H   T   A   E   D   V   N   Q   N   M   H   N   L   L   G   M   R   A   M   *

1203  TTTTAGTACTGTCATACATTTTCGTTAAATTTCAGTTGGTCTTTGACACCATGTTTAATAGCATTATGTATTCTAGGGTTCTATTATCATCAATTCCAT 1301

1302  AGTGAGTCTTTGACTTCGGTTTGGTGGCAGTAGGGCTTTCTGGAGAAAAAAAAA 1355
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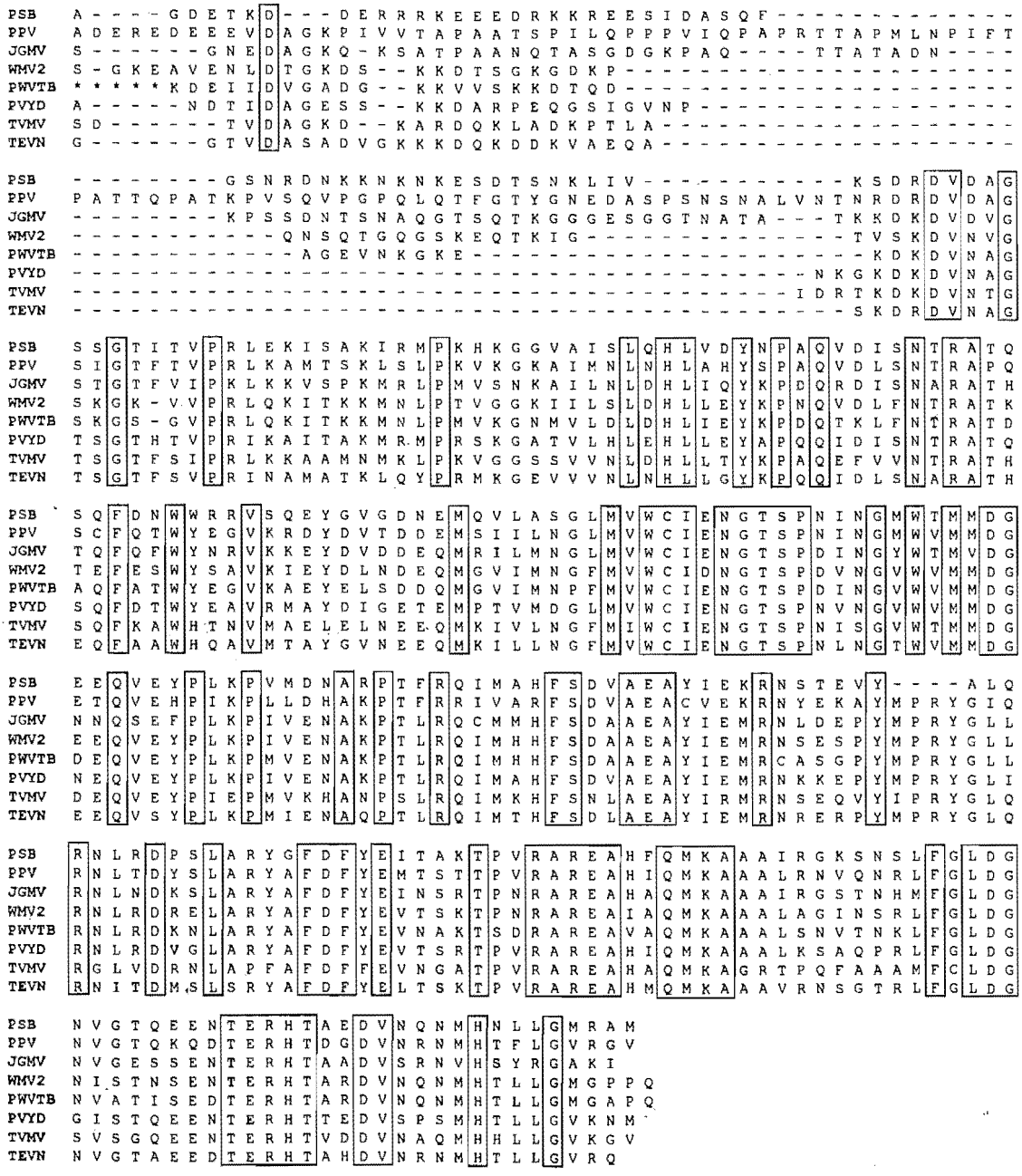
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JGMV S - - - - - - - G N E D A G K Q - K S A T P A A N Q T A S G D G K P A Q - - - - T T A T A D N - - - -
WMV2 S - G K E A V E N L D T G K D S - - K K D T S G K G D K P -
PWVTB * * * * * K D E I I D V G A D G - - K K V V S K K D T Q D -
PVYD A - - - - - - N D T I D A G E S S - - K K D A R P E Q G S I G V N P - - - - - - - - - - - - - - - -
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TEVN G - - - - - - - G T V D A S A D V G K K K D Q K D D K V A E Q A - - - - - - - - - - - - - - - - -
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TVMV - I D R T K D K D V N T G
TEVN - S K D R D V N A G
PSB S S G T I T V P R L E K I S A K I R M P K H K G G V A I S L Q H L V D Y N P A Q V D I S N T R A T Q
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